Dry powder inhalation of biopharmaceuticals: from formulation to proof-of-concept

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'Nature has its own religion'
Pearl Jam

Aan Pauline, Roderick en Lennart

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CHAPTER 1

General Introduction

Gerrit S. Zijlstra

INTRODUCTION

In the eighties of the previous century, a strong desire to administer biopharmaceuticals systemically through the pulmonary route (1-3) fuelled formulation and device development for pulmonary drug administration. After approximately 15 years of development, in 2006, the launch of Exubera® (4), the first dry powder inhalation device with a biopharmaceutical (insulin) marked a milestone. The next milestone was reached a year later when Pfizer, the company marketing Exubera®, withdrew the product from the market because according to Pfizer "it did not meet customers' needs or financial expectations", leaving Pfizer with a pre-tax loss of 2.8 billion dollar. Other commentaries were that safety issues (decrease in lung function, alleged increase of lung cancer) made physicians reluctant in prescribing Exubera®. Furthermore, the patients' conception of the relatively large inhaler and the limited dosing possibilities (only 3 an 8 IU) were mentioned as factors. Despite the bitter taste for Pfizer, the major lesson learned for the inhalation community is that any new marketed product for inhalation should hold a true (therapeutic) advantage over existing treatment and should certainly not be related to any additional safety questions compared to existing modes of administration. Next to that, the device should be convenient and robust for routine use, which now is the aim for many companies working on the development of new drug therapies for inhalation (In general, the withdrawal of Exubera® forced most companies involved in the development of biopharmaceutical products for inhalation or those working on advanced inhalation systems to re-evaluate the strategy of their development projects.

The bright side of the Exubera® case is that both Pfizer and Nektar Therapeutics, the product originator, have demonstrated that it is possible to develop an inhalable and stable dry powder product of a biopharmaceutical drug and obtain a marketing authorization; through demonstrating good formulation quality, efficient drug administration via a completely new route, clinical safety and efficacy. If the lessons described above are addressed appropriately, any new biopharmaceutical for inhalation may obtain a market authorization and be successfully marketed. Therefore, the withdrawal of Exubera® is expected to reduce research efforts to formulate biopharmaceuticals as products for inhalation only to a limited extend. Furthermore, it may shift the

activities to other drug compounds for which now an increased investment potential will become available. The interesting aspect of this shift is that the number of potential drug candidates suitable for inhalation is large and will continue to increase in the future. Next to the many biopharmaceuticals (of which several are already in development as products for inhalation), also compounds like vaccines, antibiotics, or analgesics are widely considered to be interesting drugs for pulmonary administration.

BIOPHARMACEUTICALS, FORMULATIONS AND PROCESSES

Biopharmaceuticals include amongst others nucleic acids, peptides and proteins, which are produced by extraction from non-engineered biological sources, or by other means such as extraction from engineered biological sources. The difference between nucleic acids and peptides/proteins is that nucleic acids are macromolecules build of nucleotides (nitrogenous base, a sugar, and a phosphate group) and peptides/proteins of amino acids. The distinction between peptides and proteins is arbitrary, but generally peptides rely on their primary structure (amino acid sequence) for their activity, in contrast to proteins which rely on their tertiary structure (combination of α-helices and ß-sheets) for their activity. Consequently, peptides have relatively low molecular weights, whereas proteins are of relatively higher molecular weights. Next to the above mentioned three groups the class of biopharmaceuticals includes more complex systems in which proteins or nucleic acids are contained in a specifically structured way. Examples of such systems are, whole inactivated virus vaccines, liposomes, lipoplexes or virosomes.

In practice, peptides tend to be more stable than proteins, because the tertiary structure of proteins is sensitive to changes. Pathways for deactivation are of physical and/or chemical nature. Examples of physical pathways include aggregation, precipitation, adsorption to surfaces and defolding. Examples of chemical inactivation include hydrolysis, deamidation, racemization and oxidation (6). Most deactivation pathways are related to temperature and physical and chemical stress, such as shear stress or pH. Any change in tertiary structure may affect activity, immunogenicity and/or toxicity of the protein. Therefore,

biopharmaceuticals require special attention in terms of formulation and process development and stability.

In liquid formulations, most of the mentioned degradation pathways have relatively fast kinetics as a consequence of which refrigeration is required. Furthermore, handling of the biopharmaceutical products before their use (storage, shaking) and during administration (shear forces) may further induce physical and/or chemical degradation. In contrast, many degradation kinetics are significantly slower in the dry state compared to the liquid state. This fact could even open the possibility to store the product at room temperature. However, this requires appropriate formulation and processing (drying) in order to prevent degradation of the biopharmaceutical during the production process and subsequent storage.

BIOPHARMACEUTICALS FOR INHALATION

Next to stability, formulations of biopharmaceuticals for inhalation require special attention regarding size, shape and density of the powder since the efficacy of the inhaled biopharmaceutical is related to the aerodynamic particle size. For instance, a biopharmaceutical for local pulmonary action may need a different aerodynamic particle size than biopharmaceuticals intended for systemic action administered via the pulmonary route. The appropriate aerodynamic particle size distribution depends on the desired target region within the lung and on the inhalation manoeuvre exerted by the patient, which is related to the inhalation device used. With various techniques based on pharmacokinetics, pharmacodynamics or scintigraphic techniques, attempts have been made to establish the preferable particle size distribution for various drugs for pulmonary administration. For example, *in vivo* deposition studies have confirmed that particles in the aerodynamic size range between 3 and 6 µm are optimal for deposition in the central airways (7, 8), whereas peripheral (or alveolar) deposition, typically needed for maximum systemic absorption of larger molecules (1.4 – 22 kDa), can be maximised by using particles of 1.5-3 µm (7, 9-12).

Biopharmaceuticals are designed to treat a large variety of diseases, which are of systemic or local nature. In inhalation, the lung can be used to reach four different treatment targets:

1) to treat local lung diseases (e.g. asthma, COPD or cystic fibrosis),
2) to treat systemic diseases by achieving systemic absorption (e.g. protein deficiency),
3) to obtain a systemic effect without systemic absorption (e.g. vaccination) and
4) to obtain both a local as well as a systemic effect (e.g. antibiotics in cystic fibrosis or tuberculosis)

Examples of biopharmaceuticals used to treat local lung diseases are recombinant human deoxyribonuclease I (13-17) and α1-antitrypsin (18, 19). Recombinant human deoxyribonuclease I (rhDNase; Pulmozyme®) is used in cystic fibrosis as a liquid for nebulization. RhDNase is an enzyme with a molecular weight of 32 kDa that cleaves extracellular DNA present from recurrent infections. By cleaving extracellular DNA, the viscosity of sputum decreases and thereby enables better sputum clearance and hence lowers the risk of recurrent infections. α1-Antitrypsin (AAT) is used as chronic replacement therapy in individuals who lack AAT and have an inherited form of panacinar emphysema. Lack of AAT leads to emphysema, which is marked by damage of alveolar epithelium resulting in inefficient breathing and shortness of breath. To reduce or limit the progress of emphysema, the AAT level can be restored by inhalation.
The lung can also be used as a portal to reach sufficient systemic plasma levels to treat a variety of systemic diseases. This option is often referred to as “needle-free administration”, since this administration is often considered as an interesting option to replace parenteral administration. Examples of biopharmaceuticals used to treat systemic diseases include cetrorelix (20, 21), insulin (22, 23) and human growth hormone (24). Absorption from the lung of these substances depends largely on their molecular weight. If the molecular weight is between 1.4-22 kDa, absorption can only occur via the alveolar membrane. Therefore these substances require deposition in the peripheral part of the lung. The recognition of the lung as a non-invasive port of entry to obtain systemic absorption (3, 25-27) is due to the large alveolar surface area, its extensive vascular system, thin alveolar epithelia and large intercellular pores, which not only enable effective gas exchange but also effective diffusion/transport. Beneficial over oral administration are the shorter route before absorption (resulting in a much faster onset of effect) and avoidance of the intestinal and hepatic first-pass effect (Figure 1).

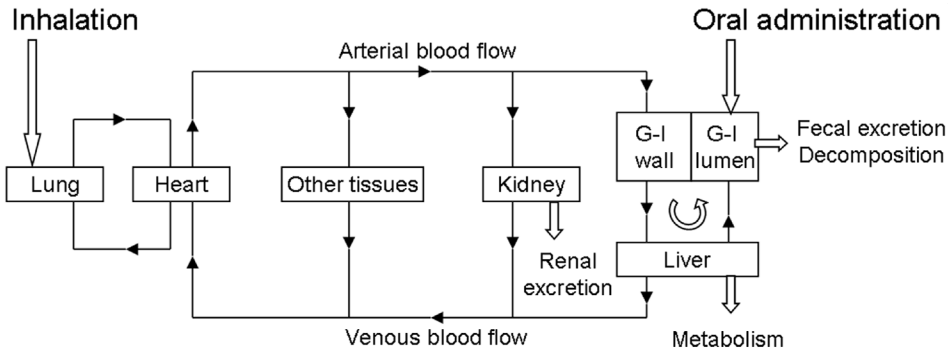

Figure 1. Schematic presentation which shows the major differences between pulmonary and oral administration (the circular arrow between gut and liver represents the enterohepatic cycle).

A further treatment objective of pulmonary administration may be to obtain a systemic effect that is reached without absorption of the biopharmaceutical product. Examples include vaccines (e.g. influenza, measles). These vaccines will not be absorbed from the lungs, but instead they will promote a systemic immune response after deposition in the lungs (28-31).
Finally, there are a number of drugs that are used via the pulmonary route to obtain both an increased local effect as well as a systemic drug level, which elicits a systemic effect of the drug. The most widely used example of this kind of use is the inhaled antibiotics in cystic fibrosis related Pseudomonas infections or in tuberculosis. In both diseases the primary infection in the lung can be effectively treated by the high drug levels in the lung resulting from the inhalation, whereas infections in other parts of the body or lung sites not directly reached by the inhaled drug are treated by the antibiotic absorbed from the lung (32-36). Also the inhalation of amphotericin B encapsulated in liposomes (AmBisome®) used to treat aspergillosis is an example of this type of pulmonary drug use (37-39). Another therapeutic area in which local and systemic levels may be beneficial is in the field of lung transplantation. Immunosuppressants, such as calcineurine inhibitors (Cyclosporine A), are given systemically to obtain a therapeutically effective level to suppress rejection. Unfortunately, calcineurine inhibitors are nephro- and neurotoxic. This toxicity leads to dose adjustments, which leads to higher chance of rejection. Ultimately, the balance between dose-related

efficacy and toxicity may lead to chronic rejection. When chronic rejection occurred, no treatment options are available next to re-transplantation. It is in the stage of chronic lung rejection where inhaled cyclosporine A as additional on-top-off therapy has demonstrated to be effective (40, 41).

For any biopharmaceutical drug product that is intended for inhalation, a variety of parameters determines its stability and efficacy. The stability of the product depends both on its qualitative and quantitative formulation as well as on the production process. However, the formulation and production process may also affect the properties of the aerosol, which in turn is a major parameter determining efficacy. Efficacy may depend on factors as particle size distribution of the aerosol, efficiency of the aerosol production, the inhaler device used and its ease of operation which in turn may be related to patient compliance. Finally, when a certain formulation and device have been developed, how do we bridge the gap between technological development and *in vivo* efficacy? Thus, for any biopharmaceutical for which a beneficiary therapeutic effect is expected upon pulmonary administration, at least three relevant questions should be addressed:

1) Which administration method available for pulmonary administration needs to be used and what type of formulation would be best?
2) Which manufacturing process should be chosen?
3) How do we test the device and formulation for safety and efficacy?

ADMINISTRATION METHODS

Various inhalation methods rely on producing an aerosol from a liquid; the liquid can either be a solution or a suspension. The aerosol can be created by disruption of a liquid jet or surface (nebulization) (42-44), pushing liquid through fine nozzles, perforated membranes or vibrating meshes (soft mist inhalers) (45, 46) or release from a pressurized canister (metered dose inhalers) (47, 48). Liquid aerosol systems generally require energy in the form of electricity or pressure to produce the aerosol.
Other methods relies on inhalation of a powder aerosol (dry powder inhalers) (49, 50) in which particles that contain the active ingredient are in the appropriate size range for inhalation. In contrast to the liquid aerosol systems, the inhalation airflow generated by the patient delivers

the energy for particle agglomerate disruption necessary to produce the aerosol.
The various currently available systems differ significantly in many aspects. Aerosol characteristics, inhalation manoeuvre efficiency of aerosol generation, lung deposition, ease of operation, maximal dose weight, dose release mode (as a bolus or gradually during a longer period) and lung deposition are just a few examples. The pros and cons of the different aerosol systems have been reviewed extensively (42-51). Pharmaco-economics may also play a role in the decision for a liquid or a dry powder formulation (52). In general, dry powder inhalation systems are considered most patient friendly because of the operability, patient mobility and device/formulation robustness. In general dry powder inhalers offer one other important advantage over the liquid aerosol systems and that is storage stability. As mentioned, powder formulations of biopharmaceuticals can be stable for long times at room temperature. Dry powder inhalers are therefore considered the preferred system for the administration of biopharmaceuticals by inhalation.

FORMULATIONS

Most biopharmaceuticals are produced in an aqueous environment. When the biopharmaceutical is going to be formulated as a dry powder for inhalation, a subsequent drying step is needed. Some biopharmaceuticals can be dried without loss of activity, such as e.g. peptides, but most will need special formulation techniques, such as sugar glass technology. To minimize loss of activity (and related increase in unwanted immunogenicity) during production and to improve storage stability. Sugar glass technology implies the stabilization of biopharmaceuticals during drying and storage by low molecular mass sugars such as trehalose, mannitol, sucrose, or a mix of a low molecular sugars with a polymeric excipients such as dextran (53, 54). This technology is the most used technique for stabilization of biopharmaceuticals. Higher molecular mass sugars, such as inulin have also been reported as good stabilizers during drying and storage (28, 29, 55). The addition of carbohydrates during drying serves two purposes:

- Introduce a physical barrier to prevent aggregation and degradation (56).
- Reduce the molecular mobility to minimize physical and/or chemical degradation reactions (57).

Two mechanisms have been proposed by which the excipients stabilize the biopharmaceutical: a thermodynamic and kinetic mechanism. The thermodynamic mechanism, also referred to as the water substitution hypothesis, states that stabilization of the native protein structure results from replacing hydrogen bonds from water by hydrogen bonds from the excipient during the drying process, thereby thermodynamically stabilizing the protein. Such an effect is unlikely to depend strongly on drying processes conditions. The kinetic mechanism proposes that stability relies on suppressing global motions, known as α-relaxations, via the formation of a rigid glassy matrix which is highly viscous. α-Relaxations mainly occur due to translational or rotational changes of the complete molecule; these motions are strongly dependent on the diffusivity. In turn, in amorphous, glassy matrices, diffusivity depends on temperature, especially around the Tg. Since global relaxation is impacted by thermal history, different drying processes and process conditions may result in different stabilities (58, 59). A typical characteristic of sugar glass stabilization is that the formulations are in the amorphous state.

In addition, formulations for inhalation require special attention since the deposition efficiency of the inhaled biopharmaceutical is related to the aerodynamic particle size of the powder. Depending on the target site in the lungs, particles in the aerodynamic size range between 3-6 µm or 1.5-3 µm are needed for central or peripheral deposition, respectively (7-12). The aerodynamic diameter can be expressed as the diameter of a unit-density sphere that has the same settling velocity in still air as the measured particle:

$$d_{ae} = d_e \sqrt{\frac{\rho_p}{\rho_0 \chi}} \quad (1)$$

where d_{ae} is the aerodynamic diameter, d_e the geometric particle size (often the equivalent laser diffraction diameter is used for this parameter), ρ_p the density of the particles (g/cm^3), ρ_0 the unit density (1 g/cm^3) and x the dynamic shape factor (1 for spherical particles). The particle density, ρ_p, in this equation is not equal to the true density of the dried material, instead the particle density is the mass of the particle divided by the volume of a sphere of diameter d_e and thus can be

significantly lower than the true density, because both internal and external voids are included.

When the biopharmaceutical is formulated as a stable powder in the size range for inhalation, the powder may need further formulation to improve flow and dispersion properties. To improve the flowability, formulations used in dry powder inhalation are either soft (spherical) agglomerates of the active ingredient with or without excipients, or (adhesive) mixtures with (carrier) excipient. Upon inhalation, dispersion of the powder to an aerosol is necessary for inhalation, which means that the soft agglomerates and adhesive mixtures need to be deagglomerated. One of the major challenges in dpi-technology is therefore to find the proper balance between the interparticulate forces in the powder mixture originating from the production process and de-agglomeration during inhalation. The forces acting between the drug-drug or drug-excipient particles have been described before (60, 61) and new approaches aim to increase the fine particle output by:

- Weakening the interparticulate forces
    - due to smoothened carrier surface, removal of adhering fines and minimize strong bonding sites
        - Surface treatment of carrier excipient particles by re-crystallisation (62),
        - Submersion in ethanol or ethanol-water mixtures (63, 64) and
        - Corrasion (65, 66).
    - Selecting the most appropriate size fraction of carrier excipient (67, 68),
    - by adding ternary excipients
        - Adding lactose fines (69, 70),
        - Adding PEG 6000, leucin or magnesium stearate (e.g. (71))
- Particle engineering techniques to reduce cohesion and increase respirability
    - Preparation of highly porous particles using different methods (72-74)
    - Supercritical fluid crystallization

All approaches have in common that they aim on increasing the fine particle output (the percentage of deagglomerated particles). In addition

several techniques aim to improve respirability of the spray dried powders by designing particles that have a low density (ρ_p) and relatively large geometric diameter (d_e). These particles are termed “large porous particles” and can be produced by two techniques: the so-called folded shells and solid foam particles (75). The term “large porous particles” was first introduced for polymer particles produced by a double- and single emulsification solvent evaporation process followed by a freeze-drying step (76). Later, large porous particles were associated with spray dried particles from a sub-azeotropic ethanol-water co-solvent system. The solid phase contained dipalmitoylphosphatidylcholine (dppc), a lung surfactant, albumin and saccharides (77-79). Due to the low aqueous solubility of DPPC and high Peclet number, DPPC acts as an early shell former. The low density arises from the low feed concentration, typically around 0.1% w/v, and the fact that the solid particle is produced at the first instance of spray drying, thus in a relatively large droplet. As a result, densities of less than 0.1 g/cm^3 are found in such particles (79). Such large porous particles showed good aerosolization performance in comparison to conventional dry powders. For instance, high emitted doses of 90-100% were reported (24, 80-82). Total lung deposition and variability were superior to conventional systems (81). However, the efficiency of deposition of large porous particles has been doubted in a different study (83) due to lower diffusion efficiency and gravitational sedimentation of large porous particles compared to submicron particles.

Folded shells are particles that contain internal and external void space caused by a soft surface layer which folds to form a wrinkled morphology. A soft surface layer can be achieved by a high initial saturation, low diffusion constant, a relatively low inlet air temperature or a combination of the three. During drying, a shell is formed which remains rubbery throughout the drying process. The internal pressure builds up due to evaporation of the solvent. When the internal pressure exceeds the tensile strength of the rubbery shell, the shell disrupts which leads to shrinking of the particle. Further drying leads to a wrinkled (raisin-like) morphology.

Solid foam particles are produced by spray drying an emulsion in which the dispersed phase consists of submicron droplets and a solvent that evaporates slower than the continuous phase. The dispersed phase is

stabilized by phospholipids, which decrease coalescence that would have occurred upon evaporation, and thus shrinking. Shrinking continues until the dispersed phase is tightly packed, and then forms a foam-like structure. This predominant method is marketed under the trade name Pulmospheres™ (84, 85). To date, a consensus on the mechanism of drying of emulsions is not reached. The dispersed phase in Pulmospheres™ usually consists of perfluorooctylbromide (86, 87), stabilized with distearylphosphatidylcholine (72, 86, 88). The phospholipid properties can be altered with calcium chloride. The active ingredient is usually dissolved in the continuous phase, but poorly soluble drugs have been suspended in the continuous phase as small crystals (88). The performance of Pulmospheres™ in combination with a passive capsule inhaler was reported to be more efficient than conventional dosage forms for dry powder inhalation (72, 87) or mdi's (86). Furthermore, Pulmospheres™ with tobramycin were reported to be nine times more efficient than nebulized tobramycin (87).

MANUFACTURING PROCESS

Several manufacturing processes are available to produce formulations of biopharmaceuticals in the appropriate size range for inhalation. These include milling, spray drying, freeze-drying, spray freeze-drying and supercritical fluid drying. The best known process to achieve particles in the appropriate size range for inhalation is milling. Freeze-drying is most widely used for drying of biopharmaceuticals, but lack of control over the particle size distribution makes a second processing step, such as milling, necessary. Unfortunately, many biopharmaceuticals are not able to withstand the forces induced during the milling process (20, 89-91). Therefore, milling has limited applicability, which reduces its applicability in inhalation of biopharmaceuticals. Spray drying, spray freeze-drying and supercritical fluid drying appear to be more suitable methods to produce biopharmaceutical powders for inhalation. These methods incorporate the drying process and provide control over the particle size distribution. These drying techniques are also used for particle engineering. Particle engineering is a discipline that uses the understanding of particle formation during drying to design particles with special properties such as low density (ρ_p) or specific shapes (x) to improve specific (inhalation) properties and/or stability. Recently, a

number of reviews were published, which give an adequate description of particle engineering techniques (75, 92-94).

Spray drying is a single step production process which is relatively simple and fast. In the past, spray drying was regarded primarily as a technique to produce particles in a certain size range and to remove solvent without increasing the product temperature to an extreme. Today, spray drying is getting more advanced through the use of special liquid compositions and excipients resulting in advanced particles, such as large porous particles (73, 76). Major advantages of spray drying are that the process can easily be scaled-up and many critical parameters are well known. Disadvantages of spray drying include shear stresses due to the atomization, heat stress caused during drying and the risk of crystallization due to use of elevated temperatures.

The spray drying process can be divided into 4 stages:
1) atomization of a feed solution into a wet aerosol,
2) aerosol mixing with air,
3) drying of the droplets at elevated temperature and
4) separation of the dried product from air.

For each step a large number of process and/or formulation variables can be applied, such as solid concentration, type of atomizer, liquid feed rate, inlet air temperature and flow rate. Stage 1 and 2 determine the droplet size distribution, which has a strong relationship to the particle size distribution of the end product. Stage 3, the drying of the wet aerosol, is the most critical step in the process because on one hand the temperature margin between drying and inducing physical and/or chemical degradation is small while, on the other hand, properties (e.g. protein concentration on the surface, morphology, specific surface area) that influence the product quality may be strongly affected by temperature and drying rate. In general, drying of the droplets is achieved by evaporation, followed by decrease in droplet size and shell formation. At a certain moment, a shell of solid material may be formed whereas the inside of the particle still contains a solution. This solvent diffuses through the particle shell and evaporates at the particle surface during further drying, leaving a solid (often wrinkled) or (smooth) hollow particle at the end of the drying process (figure 2). In a properly designed

spray drying process all parameters, such as inlet air temperature, inlet air flow rate, liquid feed rate and atomizing air flow are balanced with formulation parameters (e.g. composition or desired moisture content) and desired product properties like particle size distribution, particle morphology, yield and solid state.

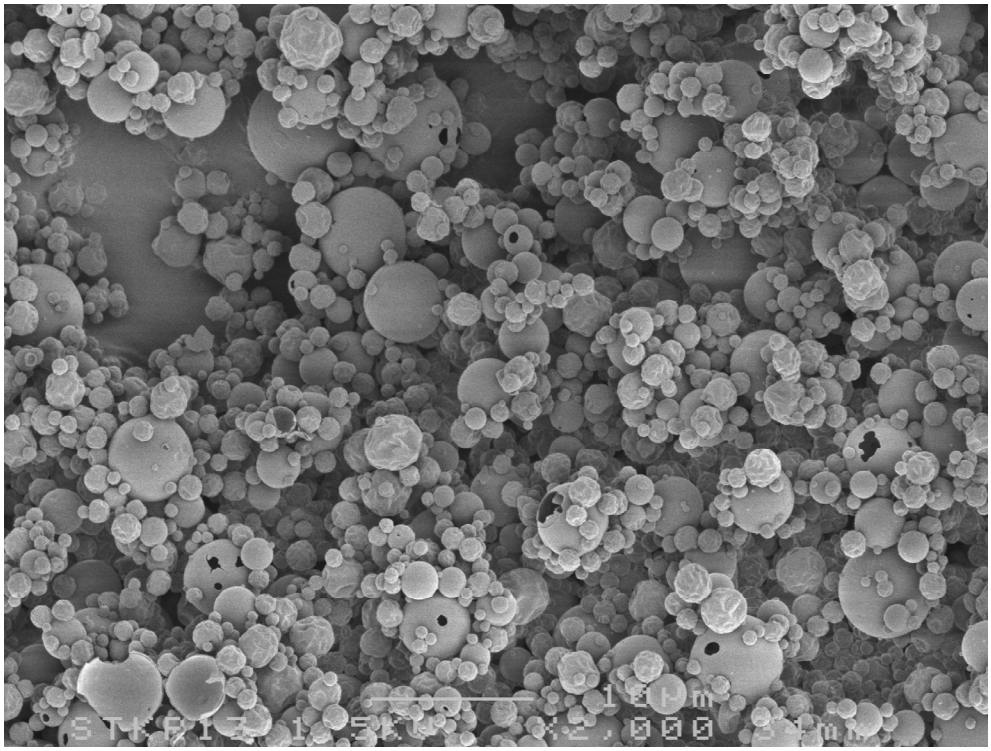

Figure 2. Scanning electron micrograph of spray dried inulin showing hollow spheres at a magnification of 2000x. Remarkably, particles with smooth surface display holes in the surface due to internal pressure build-up. The smaller, wrinkled particles apparently had a softer shell at the time of internal pressure release.

Spray freeze-drying is a process in which a fine mist is sprayed into a vessel with a cryogenic liquid, such as nitrogen, oxygen or argon. Due to the low temperature, the droplets are immediately frozen, a process that already starts in the cold vapor (95-98). The frozen droplets then settle to the bottom of the vessel as a result of their higher relative density. After lyophilization, a porous particle in the approximate size of the original

droplet remains (Figure 3) (99, 100). Advantages of spray freeze-drying include the low crystallization risk and capability to produce engineered particles. Disadvantages include exposure to shear and freezing stress, relative high costs, length of the process (which may be 1 to 3 days) and probably most important current absence of large scale pharmaceutical production facilities. However, knowledge on the spray freeze-drying process is evolving, and with the recent availability of atmospheric spray freeze-drying, which has been reported to be finished within a couple of hours, the process becomes more attractive for large scale manufacturing (101-103).

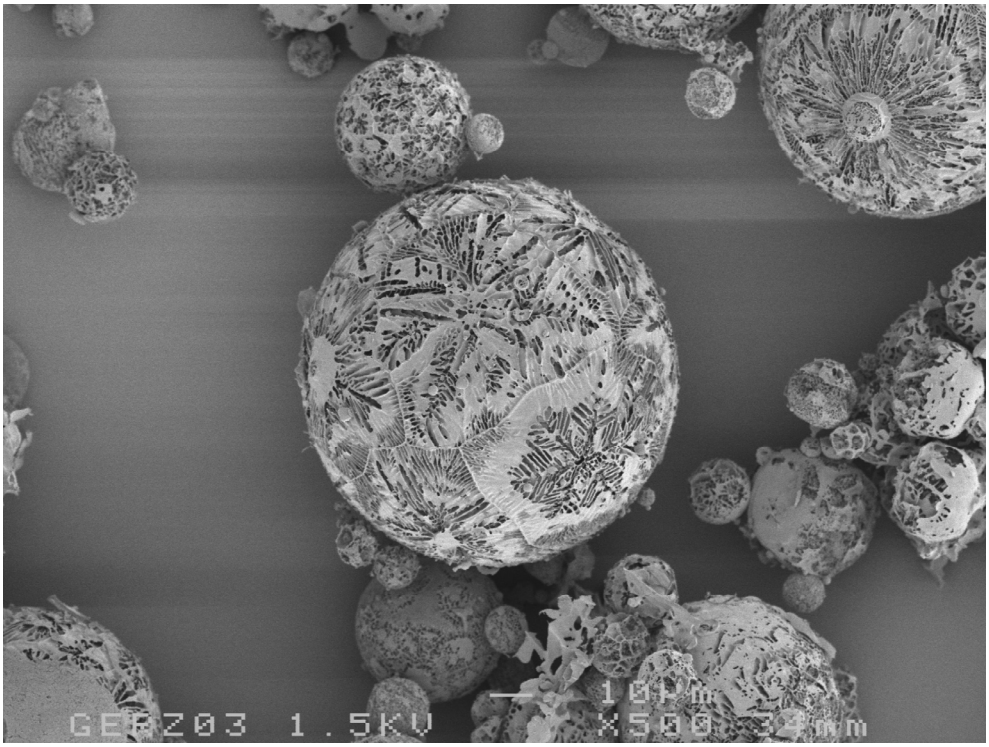

Figure 3. Scanning electron micrograph of spray freeze-dried inulin from an aqueous solution at an atomizing pressure of 400 L_n/h. Magnification was 500x. In this micrograph, the porous structure can perfectly be seen. Moreover, in the surface of the middle particle, the ice crystal structure can clearly be seen.

With spray freeze-drying, mass transfer processes during drying are completely different from those during spray drying. The most important

reason for this is the time to reach immobilization of the molecules; for spray freeze-drying this time is much shorter than for spray drying. While with spray drying the viscosity of the shell and of the droplet increases in time gradually up to a maximum upon reaching the final moisture content, high viscosities are reached almost instantaneously upon freezing of the droplet with spray freeze-drying. The whole system will be below the T_g within tenths of a second. This difference makes spray freeze-drying especially suitable for substances that show extensive phase separation, such as poorly soluble drugs (e.g. Cyclosporine A or Δ9-tetrahydrocannabinol) or very labile drugs (e.g. rhDNase, influenza subunit vaccine) (29, 99, 100).

Recently, supercritical fluid technology as a powder preparation technique for high molecular weight drugs was reviewed (104). Supercritical fluid technology is claimed to be a process with great control over particle size, shape and polymorphic purity (74, 105). Supercritical fluids (SF) are gases and liquids at temperatures and pressures above their critical points, T_c and P_c, respectively. In pharmaceutical applications, CO_2 is generally being used as supercritical fluid ($SFCO_2$) due to the low T_c (31.2 °C) and P_c (7.4 MPa). Of particular interest are the conditions at which the supercritical fluid is present as a single phase but with advantageous properties of both liquids and gases. These conditions are met within the range of $1<T/T_c<1.1$ and $1<P/P_c<2$ (93). In these ranges, the density is such that solvation is enabled, while the viscosity in supercritical fluids is lower than in solution. As a consequence, the diffusivity is higher, which facilitates effective mass transfer. Of great practical use is the high compressibility of supercritical fluids, with which the density and thus solvation power can be controlled. Advantages include process speed and the possibility to produce engineered particles. A major disadvantage is that supercritical fluid technology is still in development, and thus has a limited knowledge base and relative high costs. Another disadvantage is atomization-induced shear stress. Despite its infancy, supercritical fluid technology receives a great deal of research interest. For example, process conditions such as type of nozzle (106), flow rate (107), type of modifier (108) and modifier (ethanol) faction (109, 110) have been investigated on functional outcomes as polymorphism (107, 111) and particle size/morphology (110). The fraction of the modifier ethanol plays role in polymorphism (111) and

storage stability as residual ethanol lowers the Tg (109). Different modifiers as methanol, 2-propanol or acetone may also be used (108). For protein stabilization, regular carbohydrates such as e.g. dextran, chitosan, maltose, sucrose and trehalose (109, 112-116) have been used for stabilizing proteins. As any other drying technique used for stabilization of biopharmaceuticals, supercritical fluid technology is likely to result in tailor-made process/formulation approaches, as illustrated by Jovanovic, who needed to tailor the process that was successful for processing lysozyme to a process suitable to produce a stable dry powder formulation of IgG with the right quality attributes as structure, aggregation and solubility (116, 117). In animals, chitosan proved to be a functional excipient, not only in stabilization of for example insulin (114), but also to improve the bioavailability in rats when using a chitosan type, with a degree of quaternization of 60% (DQ60) , to a level almost similar as a subcutaneous injection. Chitosan DQ60 functions both as a mucoadhesive and a permeation enhancer (112). A combination of chitosan and Diphteria toxoid also demonstrated to be at least equivalent to subcutaneous injection in immunological response and neutralizing antibody titers (115). All these examples together demonstrate a proof of concept, showing that although this manufacturing method is still in its infancy, there may be a successful future for supercritical fluid technology.

Each method thus has its own typical advantages and disadvantages. The relevance of the different disadvantages may be determined by the characteristics of the biopharmaceutical, the formulation and process parameters. Because of the huge knowledge base, long-term industrial experience and ease of the process, spray drying seems to be the best choice to produce a powder for inhalation of biopharmaceuticals. This may explain why spray drying received most attention in literature as drying method for proteins in inhalation (figure 4).

In summary, many biopharmaceuticals may be produced to a powder for inhalation by using spray drying. In a limited number of cases, other manufacturing methods may be needed, for example when the stability of the biopharmaceutical is such that it cannot withstand the stresses caused by spray drying. Alternative methods are then spray freeze-drying and supercritical fluid drying.

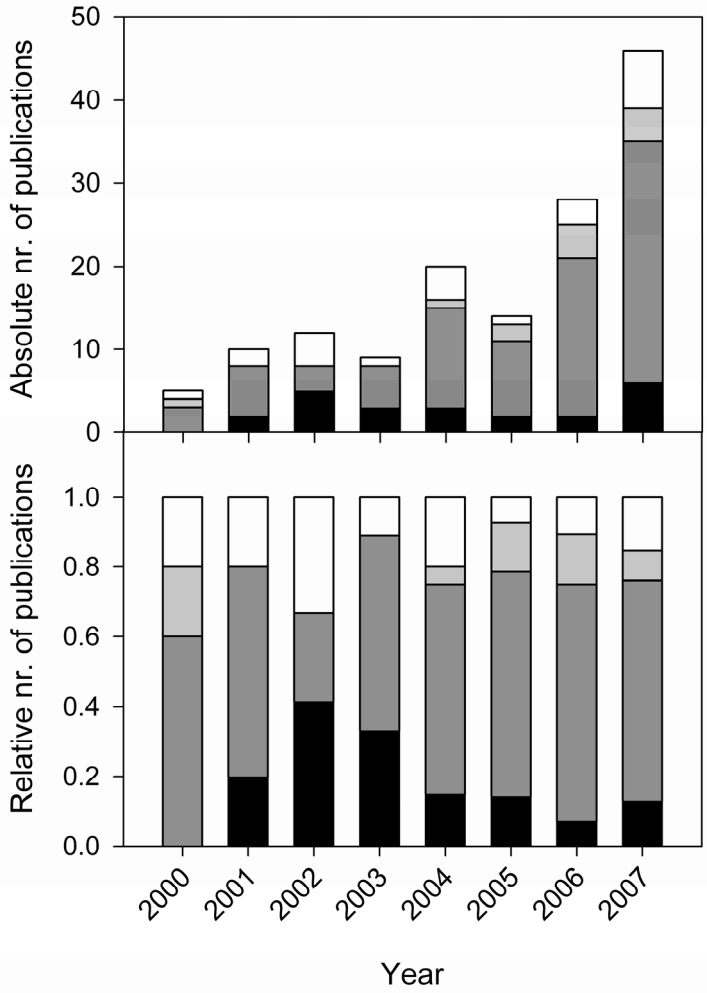

Figure 4. Number of publications (A: absolute; B: relative ratio) per year on milling (black bars), spray drying (dark grey bars), spray freeze-drying (light grey bars) and supercritical fluid technology (white bars). Search terms were "inhalation" and "milling", or "spray drying", or "spray freeze-drying", or "supercritical" in the title in the Pubmed database.

BRIDGING THE GAP BETWEEN FORMULATION TECHNOLOGY AND *IN VIVO* EFFICACY AND SAFETY

In the development of innovative medicinal products animal models are essential to provide indispensable knowledge on absorption, distribution, metabolism, excretion and the related toxicology, safety and efficacy. An animal model is an intact animal preparation that has a defined scientific

utility (118). In addition, animal models may be used to determine biopharmaceutical aspects such as the deposition characteristics of inhalation medicines and serve as a comparative *in vivo* screening method for different formulations. A common problem in all animal models used in inhalation research is the question how the drug should be administered to have the best extrapolation to humans and which animal could best be used.

Understanding the differences in airway anatomy between animals and humans is important for adequate extrapolation between species. Most obvious are the differences in nasal anatomy (119, 120), but the tracheo-bronchial tree and the deep lung region differ also (119-123). Humans have a relatively simple nasal anatomy, while other mammals have a complex internal structure with a large olfactory apparatus (119, 120). In addition, rats and possibly other rodents are obligatory nose breathers, incapable of mouth breathing (124). This property enables their use in nose-only exposure models as will be detailed below. The complex olfactory apparatus limits the probability of inhaling particles larger than a few micrometers (118).

In humans and some animals (dogs, cats, monkeys and ferrets), the transition from cilated tracheobronchial airways to fully alveolated alveolar ducts is gradual and several orders of respiratory bronchioles are present (122, 123). In contrast, in mice and rats the respiratory bronchioles are absent while in other animals such as hamsters, guinea pigs, oxen, sheep and pigs respiratory bronchioles are very abbreviated (122, 123). An implication of having respiratory bronchioles is a slower alveolar clearance (125). In addition, with increasing body size, the alveolar size, number alveoli, and total lung surface area increases (126).

Information about comparative deposition studies is scarce even though particle deposition in laboratory animals has been reviewed (126, 127). In general, humans have a larger peak particle size for pulmonary deposition than dogs, guinea pigs, monkeys and rats. The highest deposition rate in the tracheo-bronchial tree and alveolar region is obtained with particles smaller than 1 µm in rodents, in dogs with particles between 1 and 2 µm and in humans between 3 and 5 µm. The total deposition efficiency of particles having an aerodynamic particle size between 1 and 5 µm for

humans, dogs, guinea pigs and monkeys is roughly similar, but lower for rats (124, 127-130).

Generally, the following administration methods are available to administer aerosols to animals. These methods can be divided into passive and active administration methods, referring to the principle by which the aerosol is brought into the lung.

- Passive methods (animal inhales aerosol by breathing)
  - Whole body chambers
  - Nose-only exposure
- Active methods (animal gets aerosol administered)
  - Intratracheal instillation
  - Intratracheal insufflation

The passive methods are divided into whole body chambers and nose-only exposure. A benefit of using passive methods is that the aerosol is inhaled in a physiologic manner, enabling research on the lung penetration and deposition of the aerosol (e.g. the effect of particle engineering). In addition, the complete respiratory tract is covered and the animal does not have to be anaesthetized. A disadvantage of passive methods is that the inhaled dose cannot be adequately controlled. Since the animals need a number of breathing cycles to inhale a substantial dose and the dose is to be given over a longer period of time (131).

With whole body chambers, the animal generally is unanaesthetized and unrestrained. An aerosol is externally created and transferred to the aerosol chamber, where the animals may inhale the aerosol. This method is generally used for small laboratory animals, such as mice, rats and guinea pigs. Sophisticated method development of the whole body chamber concept has led to amongst others a model that allows investigation of chronic asthma and airway remodeling (132). A specific disadvantage of whole body exposure is that the aerosol will also deposit on the fur. After licking the fur, the drug may be absorbed via the oral route, making possible effects less attributable to inhalation. To reduce possible absorption through the oral route, nose-only exposure models have been developed. With nose-only exposure, the animals are fixated in restrainers to allow adequate positioning in an orifice that is connected to

a chamber containing the aerosol. In this way, only the nose is exposed to the aerosol, thereby reducing fur deposition. Such nose-only methods were mainly developed for liquid aerosols or smoke exposure (131, 133), but can also be used for dry powder aerosols (134).

The active methods have as benefit that they allow precise dose metering and that the dose can be given in a bolus, this enables for example dose-response measurements. Furthermore, the method is relatively simple, even though transoral intubation is needed. Fur deposition is not an issue with the invasive methods. Disadvantages include that the method is non-physiologic and that the upper respiratory tract is avoided. Furthermore, anesthesia is required for the intubation procedure. Anesthesia potentially may alter clearance rates and influence the effects (135). Two methods different active administration methods have been developed: intratracheal instillation and intratracheal insufflation. The difference between the two is that instillation is performed with a liquid(21) and insufflation with a liquid or powder aerosol (29). Important factors to consider with intratracheal administration are the intubation method, the administered dose and the method of anesthesia. For instillation, additional factors are the vehicle to suspend or dissolve the drug in and the total volume used. Intratracheal administration is widely used due its relative simplicity, low cost and the accurate dose metering. The intubation procedure requires skilled personnel.

How do passive and active methods relate in terms of deposition patterns, absorption and clearance? First of all, the dose administration rate is different for the two methods: with active methods the dose is given as bolus, while with passive methods dosing takes a certain period of time encompassing a number of inhalation cycles. In addition, with passive methods, the upper respiratory tract is also exposed to the aerosol in contrast to the active methods. Therefore, with passive methods the deposition pattern relies more on the aerodynamic particle size than with active methods. The deposition pattern obtained with passive methods is more homogeneous than obtained with active methods. There are indications that by using active methods, more “patchy” deposition patterns are observed, which may have a consequence for clearance, the site and degree of systemic absorption and local toxicity (131, 135-138). For example, clearance by macrophages

has been described to be dependent on the administration method (138). With active methods, a large variation could be observed in foreign material load of macrophages, with some containing no foreign material while other macrophages were overloaded. In contrast, by using passive methods, the macrophages contained a relatively similar amount of foreign material. Therefore, the different methods have their own distinct pros and cons. In practice, the choice for any administration method will often rely on the type of study (dose needed, length of study, type of drug, desired lung-deposition profile) and the available expertise in aerosol administration to laboratory animals.

Through experimental handling, a specific disease can be developed in animals. Such animal models do not only enable improved understanding of certain diseases but also the conduct of efficacy studies in animals. For example, animal models have been developed for tuberculosis infection (139, 140), chronic obstructive pulmonary disease (133, 141), asthma (142, 143), cystic fibrosis (144) and lung transplantation (145). Some animal models only require inhalation of toxic or infectious substances (COPD and tuberculosis model) while other models require gene manipulation (cystic fibrosis) or surgery (lung transplantation). By combining such animal models with a certain administration method, early proof of principle or proof of concept can be generated. Such studies are needed to bridge the gap between formulation development and first trials in humans and are for ethical reasons essential.

CRITICAL NOTES

In the field of inhalation (of biopharmaceuticals) several aspects are still the subject of intense discussions and conflicting opinions. A major point of discussion focuses on what the best device and/or formulation for a specific patient would be. Irrespective of the device used the patients characteristics (age, lung function, disease status, compliance) determine to a large extend the choice for an inhalation system and essential characteristics of the inhalation such as the extend of lung deposition as well as location of the deposition (predominantly central or peripheral). On the other hand, the type of formulation (liquid or dry, powder production technique which affects size and density) and the combination with a certain device (nebulizers, pressured metered dose inhalers, passive or active dry powder inhalers) determine also the aerodynamic

particle size distribution and thereby deposition characteristics. Ultimately, only an in-depth evaluation of the complex of relevant parameters in the context of their mutual interrelationships may lead to an optimal choice for the therapy, formulation and device. Some major parameters playing a role in this complex are:

- Patient characteristics (lung function, age, disease state, compliance, etc.)
- Desired lung deposition
- Deposition target in the lung,
- Physico-chemical characteristics of the drug and the formulation
- Type of device

However, the set of parameters given above is far from complete, and it should be realized that the simple optimization of just one parameter, may too often not lead to an optimal therapeutic result. The best available therapy is not always that device-formulation combination that would result in the highest fine particle fraction, but it is the combination of patient (age, lung function, disease status, compliance) with a certain device-formulation combination that is effective. For instance, a dry powder inhaler is not effective in children younger than 5 years old. Instead, often a combination of a MDI with valved holding chamber is used. Therefore, the primary determinant to choose a certain formulation and/or device depends on the patient, whose characteristics cannot be controlled. It should therefore be appreciated that general improvements on formulations and devices will bring highly differentiated improvements in therapy for different patient groups.

The strategies thought of that improve the formulation and device are the following. First, the formulation needs to be such that the powder is easily dispersible into the primary particles. In addition, the batch-to-batch variation of the primary particle size needs to be very small to ensure that the physical properties of the powder are constant. Monodisperse aerosols are also under investigation for claimed targeted deposition. Most of the improvements are found in the devices and relate to improvement of the fine particle fraction, reduction of coordination problems and improvement of the deep lung deposition. To improve the fine particle fraction, the device should reproducibly produce the fine particle fraction, which preferably is dependent on the patients' inhalation characteristics. Some devices are under development that use

auxiliary energy to improve the fine particle fraction, and some of them adapt the aerosol generation to the breathing pattern of the patient to reduce coordination problems. To further reduce coordination problems, the dose should be given after breath actuation. The time in which the dose is released should be within 0.5 second to improve deep lung deposition. The particle velocity from the inhaler should be reduced to minimize oropharyngeal deposition (loss) in favor of deep lung deposition (51). Devices that improve patient compliance and reduce healthcare costs are easy and robust to use (light, easy to carry and not require electricity or pressurized air), give feedback on the success of the inhalation maneuver and contain a multidose compartment with dose counter (51).

Most new inhalers have been described to exercise insufficient control over the flow manoeuvres (12), which could lead to a variation in deposition patterns. To decrease the influence of variability in inspiratory air flows, the inhaler resistance can be increased (146). However, the acceptance of high resistance dry powder inhalers is still low as it is believed that patients with strongly reduced lung function may find it difficult operate such devices successfully (147), which is a widespread misconception. High resistant inhalers are generally efficient delivery (and dispersion) devices (12) due to higher turbulence compared to low resistant inhalers at a certain air flow and the fact that different dry powder inhalers tend to be operated at the same respiratory effort (148). As a result, they are capable of producing high fine particle doses already at relatively low pressure drops (149-151). Even when being operated at the same pressure difference, the amount of work (product of pressure drop, volumetric flow rate and inspiration time) delivered by a patient to inhale the same volume remains similar when the resistances differ as much as between the Aerolizer ($R = 0.019\ kPa^{0.5}.min.l^{-1}$) and the Inhalator Ingelheim ($R = 0.044\ kPa^{0.5}.min.l^{-1}$) when operated at comfortable inspiratory effort (148). Besides that, patients tend to generate the highest pressure drops through the highest air flow resistances (146).

The performance of so-called passive dry powder inhalers (dpi's) has been criticised in respect of undesired dependence on the patient's inspiratory flow (12, 72, 152, 153). Whether this is really a disadvantage or not, may depend on the desired site of deposition and the extent to which the fine

particle dose is affected by the inhalation flow rate. Considering that the deposition of particles shifts to higher airways when the flow rate is increased (e.g. (154)), it may be expected that a more constant therapy is obtained when the fine particle dose is balanced with the inhalation manoeuvre, which means that more particles have to be delivered at higher flow rates to compensate for higher losses in the upper respiratory tract. There is not much evidence in literature for this expectation, as most marketed devices up to now produce a more or less constant fine particle dose at all flow rates (155). As a result of that, the manufacturers have no interest in showing the clinical effect as function of the inspiratory flow rate. Yet, there are some indications which seem to prove the importance of balancing. Nelson *et al.* (156) showed for the Spiros dry powder inhaler with battery powered dispersion system, delivering a flow rate independent fine particle dose, that lung deposition of beclomethasone propionate decreased from 41 to 30% (and oropharyngeal deposition increased from 37 to 54%) when the flow rate was increased from 15 to 60 l/min. Also Usmani *et al.* (7) reported for monosized salbutamol particles of different sizes a strongly decreasing aerosol penetration into the lung when the flow rate was doubled from 31 to 67 l/min. As to be expected, the difference was greatest for the largest particles. In line with these findings, Newman *et al.* (157) found for the Novolizer, which produces a very fine aerosol and exhibits an increasing fine particle dose with increasing flow rate (150), that peripheral lung deposition may almost be the same at all flow rates (between 45 and 90 l/min). The flow-independent deposition behaviour is explained by the higher fine particle dose generated at higher inspiratory flows, which compensate the increased inertial impaction in the upper respiratory airways caused by the higher inspiratory airflow (51).

Monodisperse aerosols are claimed to consist of particles of the same size. The geometric standard deviation (GSD) ideally has unity, but practically a value below 1.22 is considered to describe a monodisperse size distribution (158). Monodisperse aerosols are thought to have greater specificity of airway targeting than polydisperse aerosols (159). However, deposition modelling studies show that particles of uniform size are deposited in all airway generations, with peaks in fractional deposition that vary with the inspiratory flow manoeuvre (e.g. (154)). The effect of flow rate and inhaled volume is even different for particles of different

size (160), particularly when oropharyngeal deposition is taken into account. This makes comparing the deposition efficacies of monosized particles with different aerodynamic diameters rather arguable when the inspiration manoeuvre is not precisely controlled with respect to flow rate, duration and inhaled volume. Besides, deposition is also influenced by lung morphology (airway dimensions) and local obstructions (161), factors that are uncontrolled and highly variable from patient to patient. It is thus easy to understand that it will be impossible to define just one optimal aerodynamic diameter that can be applied for targeting to a specific site of action for the whole population, even when the inspiratory flow conditions are fully controlled and precisely the same. From this point of view it may be questioned whether polydisperse aerosols in spite of their lower specificity, are not better to use since they at least will always result in some deposition at the site of action.

Finally, several dry powder inhaler systems are in development to replace wet nebulization, for instance in antibiotic therapy for cystic fibrosis patients (87, 151, 162). Although the advantages of dry powder administration seem to be clear and a significant improvement of CF therapy is expected from this replacement in administration systems (163), many aspects still remain uncertain. There is for instance a difference between single inhalation (as from a dpi) and drug delivery during several breathing cycles at tidal breathing level (as during nebulization). From the total lung volume of (adult male) humans (approx. 6 L) and the residual volume (1.2 to 2.5 L), it can easily be calculated that the maximal refreshment of air is 3.5 to 4.8 L (60 to 80% of total lung volume). Practically, inhaled volumes through dpi's are often considerably lower, as patients often exhale insufficiently before taking a dose. Particularly for COPD patients and older asthmatics inhaled volumes may be confined to only 1.5-2.5 L. This has consequences for the penetration depth of airborne particles, particularly when the entire aerosol is not released as a bolus at the beginning of the inhalation manoeuvre. Taking into account that a laminar air flow exists at all flow rates beyond generation 5, it may be expected that mixing of residual air and newly inhaled air (containing the aerosol) during a single inhalation is rather incomplete. Therefore, inhaled volumes of 1.5 to 2.5 L (corresponding with 25 to 40% refreshment) may reach no further than generation 19 and 21 respectively. When the entire aerosol is

homogeneously distributed in the first litre of inhaled air, 50% of the dose may travel not further than generation 17 to 19. This has the consequence that a major portion of the airborne particles which possess the potential to enter the alveoli on the basis of their aerodynamic properties, will not get there. This, in contrast with wet nebulization over several inhalation cycles during which mixing by convection and diffusion will be more complete. High doses of dry powders inhaled in one single manoeuvre may also cause a different response in the patient (e.g. cough and chest tightness) than wet aerosols administered at much lower concentrations over a longer time; this may also depend on the (physico-)chemical properties of the drug. It may therefore be questioned whether spreading a high powder dose over a small number (2-3) of subsequent inhalations burdens the patient less and will be more effective than inhaling the entire dose in only one manoeuvre.

For biopharmaceuticals, much effort has been devoted to advanced particle engineering. One of the developments is the use of so-called large porous particles that have densities of less than 0.1 g/cm^3 (79). The relevance and benefit of using large porous particles is claimed to be improved dispersion characteristics and improved aerosol behaviour and deposition characteristics (76, 79, 81). However, improved dispersion characteristics can also be achieved by effective inhalation devices, such as those with high resistance (149-151). In addition, despite total lung deposition and variability of large porous particles were superior to conventional systems (81), the efficiency of deposition of large porous particles has been doubted in a different study (83) due to lower diffusion efficiency and gravitational sedimentation of large porous particles compared to submicron particles. Finally, surface characteristics such as specific surface area and surface concentration of the drug may affect stability of the particles. Especially different drying methods may result in different surface characteristics. For instance, the specific surface area of particles prepared by spray drying or spray freeze-drying differ largely (20, 99). Some studies have demonstrated that particles with higher specific surface areas demonstrated inferior storage stability compared to similar formulations with lower specific surface areas even though secondary structures of the molecules were similar for the different drying methods (98, 164). In addition, the surface concentration was also reported be of influence on storage stability. For instance, a

formulation of recombinant human interferon-γ (rhIFN-γ) that was spray freeze-dried had a higher protein concentration on the surface and lower stability compared to the freeze-dried formulation (98).

CONCLUSION

To produce inhalation formulations of active substances many particle engineering techniques may be used, next to a variety of formulations and devices. Recent trends in inhalation product development can be summarized by maximization of lung deposition by increasing the fine particle dose, improvement of particle characteristics with regard to both the aerosolization behavior as well as the stability of the powder and the development of inhalation devices that enable more effective inhalation as well as a more comfortable inhalation. The increasing number of biopharmaceuticals has fuelled research both in formulation technology and device technology. However, many of the research is still performed on small scale and little has been picked up by the industry. Moreover, much of the research focuses only on one or two relevant parameters and tends to neglect the interacting other parameters determining the success of an inhalation therapy. So far, the first inhalable insulin product, Exubera®, made it to the market but the product was withdrawn by the company within one year after market introduction. Despite the bitter taste for Pfizer, the lesson learned for the inhalation community is that any new marketed product for inhalation should hold a true therapeutic advantage over the existing treatment and be free from any doubts regarding safety compared to the conventional therapy. Moreover, the device should be convenient and robust for routine use. Finally, in many European countries a large emphasis is placed on the costs for medicines. In short, the cost for a medicine to save one life year should in general not exceed the benefit of that life year. Therefore, for a successful launch of a product for inhalation, formulation research and device development should go hand-in-hand with regular cost evaluations. Finally, although the current investments in the development of new inhalation therapies is high and it is easy to imagine a multitude of potential improvements and advantages of these therapies. It should be realized that due to the high attrition rate in the pharmaceutical industry only a small number of the developments discussed in this chapter will reach the market.

AIM AND OUTLINE OF THIS THESIS

Next to Exubera®, there are very few biopharmaceuticals products for inhalation (only Pulmozyme®) that have reached the market. There are various therapeutic possibilities, not only for new substances, but also for improvement of current therapy with existing substances. The research described in this thesis focuses on contributing to knowledge on pharmaceutical aspects of inhalation of biopharmaceuticals. Emphasis was placed on dosage form development (especially dry powder inhalation systems) and on preclinical efficacy and safety investigations. The research was based on a number of model compounds that each had their own characteristics and problems.

In the first phase of the research, systemically acting peptides were investigated for which only formulation aspects were relevant, since their absorption after pulmonary administration was already proven. The first peptide investigated was cetrorelix. For this peptide, only the formulation research had to be carried out, since aspects related to efficacy of the pulmonary administration were already investigated. A range of different formulation techniques was evaluated to find the best formulation for use of the product in the Novolizer® inhaler. The second peptide investigated was cyclosporine A. Because the pulmonary use of this drug with nebulizers had failed in clinical studies, probably because of formulation related side-effects, it was decided to develop an innovative dry powder formulation of this drug. However, this approach not only required formulation development but also the development of an adequate lung transplantation model in animals to test efficacy and safety.

Subsequently, a larger locally acting protein (rhDNase) was chosen as subject for formulation research. Next to that, also pharmacoeconomic aspects of the newly developed product were investigated.

Finally, a highly innovative approach was chosen in which the therapeutic protein was not administered by inhalation, but its endogenous synthesis was induced by a small organic substrate that was inhaled. A major advantage of this approach is that the formulation of organic compounds is much easier than formulating proteins as powder for inhalation.

In chapter 2, the role of particle engineering techniques with respect to *in vitro* aerosol behavior of the decapeptide cetrorelix, a GnRH-antagonist used for ovarian super stimulation and intended for systemic

administration, was investigated. Cetrorelix was milled, spray dried, spray freeze-dried and subsequently formulated as an adhesive mixture. The effect of inhaler design (deagglomeration principle) on aerosol properties in relation to the differently produced particles was also investigated.

In chapter 3, the lipophilic cyclic undecapeptide cyclosporine A was spray freeze-dried to obtain an inulin-based glassy solid dispersion for inhalation. Several pharmaceutical aspects of this product were investigated. *In vitro* aerosol characteristics were determined, but also the inclusion and molecular arrangement of Cyclosporine A in the inulin glass was studied with Fourier Transformation Infrared spectroscopy.

In chapter 4A, the inulin-based solid dispersion of cyclosporine A was tested in an *in vivo* left lung transplantation rat model and compared to orally administered Neoral®. In this study, the cyclosporine A solid dispersion was administered with the PennCentury insufflator to obtain intrapulmonary administration. First, the pharmacokinetics of a single 10 mg/kg dose of cyclosporine solid dispersion and Neoral were determined. Next, the efficacy and safety of the cyclosporine A solid dispersion and Neoral were investigated.

In chapter 4B, the safety of the cyclosporine A solid dispersion, the excipients and the administration technique were further investigated and compared to healthy control animals.

In chapter 5, the choice of excipients (sucrose, trehalose or inulin as stabilizer) and powder preparation technique (spray drying or spray freeze-drying) to produce a stable and inhalable amorphous glass formulation of (recombinant human) deoxyribonuclease I was investigated. The best performing formulation in terms of stability and *in vitro* inhalation behavior was subsequently tested for its efficacy of reducing the viscoelasticity of sputum from 5 cystic fibrosis patients.

In chapter 6, pharmacoeconomic aspects of different modes of pulmonary administration of recombinant human deoxyribonuclease I, a 33 kDa protein were investigated. Factors such as the costs of the medicine, the deposition efficiency and patient compliance on total costs were

calculated and Pulmozyme® was compared to the dry powder inhalation product.

In chapter 7, the organic molecule ferriprotoporphyrrin (hemin) was formulated as a powder for inhalation to induce heme oxygenase synthesis in the lung. The heme oxygenase in turn is able to attenuate inflammation by virtue of its end-products ferritin, bilirubin and carbonmonoxide. First, spray drying as a powder preparation method was evaluated. Furthermore, a non-invasive, nose-only exposure chamber was developed to administer powders for inhalation to small rodents such as mice and/or rats. Finally, the efficacy of the pulmonary administration of hemin was evaluated in mice.

In chapter 8, the findings of the studies described in this thesis form the basis for some concluding remarks and a discussion on the perspectives for future research in the field of the pulmonary administration of biopharmaceuticals.

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CHAPTER 2

The role of particle engineering in relation to formulation and de-agglomeration principle in the development of a dry powder formulation for inhalation of cetrorelix

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ABSTRACT

We formulated cetrorelix acetate, as an adhesive mixture for use in dry powder inhalation. To achieve the highest possible deposition efficiency we investigated both the influence of different micronization techniques and different inhalers. The Novolizer with an air classifier as the powder de-agglomeration principle and the ISF inhaler were used for in vitro deposition experiments (cascade impaction). Micronization by milling as the classical approach and micronization by spray drying and spray freeze drying as advanced particle engineering techniques were investigated to determine whether advanced techniques are necessary to obtain high fine particle fractions (FPF) for this specific drug. It was found that the effects obtained with a certain micronization technique depended on the complex interaction of the physical characteristics of the drug substance with the type of formulation chosen, as well as with the de-agglomeration principle used. The combination of particle engineering by spray drying and the use of the air classifier technology resulted in a fine particle fraction of 66%, while spray freeze drying yielded extremely fragile particles resulting in a FPF of only 25%. The behaviour of the milled material showed similar trends as the spray dried material but FPF values were lower. It was concluded that when a drug is to be formulated as a powder for inhalation with high fine particle fractions, it is profitable to use advanced particle engineering techniques, however the applied technique should be tuned with the characteristics of the formulation type and process as well as with device development.

INTRODUCTION

Cetrorelix acetate, for convenience referred to as cetrorelix, is used for the controlled ovarian super stimulation for assisted reproductive technique in a 0.25 mg dose (multiple dose regimen). However, there are more therapeutic opportunities for this decapeptide, which are captured under the term “sex-hormone dependent pathologies” (1, 2).

Cetrorelix, a decapeptide, is currently used in a subcutaneous dosage form. For systemic administration, the pulmonary route offers opportunities as well as great advantages compared to other non-invasive routes of administration (3-8). The large surface area of the lung (approximately 40 – 100 m^2), thin alveolar epithelia, the extensive vascular system, and avoidance of the first-pass effect contribute to the expectation that the pulmonary route is the most promising non-invasive route by which (deca-) peptides can be administered to obtain systemic absorption (9-11).

Lizio et al. showed that after pulmonary administration of a liquid cetrorelix formulation systemic absorption occurred, demonstrating the suitability of this route of administration (1, 2, 12). However, for widespread use dry powder formulations may be advantageous (13, 14).

The aerosol of a drug for dry powder inhalation should preferably have a high fraction of particles in the aerodynamic size range of 1 – 5 µm (fine particle fraction) for effective delivery to the lung (14, 15). To obtain a powder in the required size distribution, several techniques can be applied (14, 16). These techniques include standard techniques such as milling (14, 17), and more advanced techniques such as spray drying (18-20), spray freeze drying (21, 22), super critical fluid extraction (23, 24) and crystallization (25). Optimization of the fine particle fraction has often been sought in either advanced particle engineering to generate particles with improved aerosolization behaviour (e.g. large porous particles, PulmoSphere) or solutions to overcome the increase of surface free energy which is inherent to milling (26-28).

The aim of this study is to formulate cetrorelix with a high fine particle fraction as a powder for inhalation using three different methods for micronization, namely milling, as an example of a standard technique, and spray drying and spray freeze drying as examples of more advanced techniques applied in particle engineering. We investigated the effects of the three methods on shape, size and density in relation to the aerosolization behaviour, in order to decide which micronization method

delivers the particles that are most suitable for inhalation using the adhesive mixture type of formulation. For dispersion of the formulations we used the Novolizer dry powder inhaler (29, 30), and the ISF inhaler (31). De-agglomeration in the Novolizer is mainly based on inertial forces occurring in the air classifier which results in high de-agglomeration forces. In the ISF inhaler de-agglomeration is based on lift and drag as well as inertial forces. These two inhalers were used to test the hypothesis that both the types of particle engineering technology applied in the formulation as well as the efficiency of the de-agglomeration principle are relevant to the performance of the dry powder inhalation system (generation of the fine particle fraction).

MATERIALS AND METHODS

Materials

Cetrorelix acetate was obtained from Sofotec (Frankfurt, Germany) in the form of a lyophilisate. The lyophilisate was used as raw material for the comminution studies.

Small batches (50 gram) of lactose, size fraction 63-90 µm, were derived from Pharmatose 100M (DMV International, Veghel, The Netherlands) by 20 min. vibratory sieving (in a Fritsch Analysette 3, Idar Oberstein, Germany) followed by 10 min. air jet sieving (in an Alpine A200 LS, Augsburg, Germany).

Methods

Preparation techniques for cetrorelix

Milling

The milling of cetrorelix was performed with a pearl-mill equipped with a cryostat. The system was cooled to -60°C and 5 gram of cetrorelix lyophilisate in 243 ml HFA 227 was milled with 100 ml ZrO_2-Ir stabilized pearls (diameter = 0.6 mm) for 15 min. Using this process, only minute amounts of degradation products (<1.17%) were found, which is indicative of the stability of cetrorelix (17). Furthermore only minimal metal contaminants (Zr = max. 0.6 ppm; Fe, Cr, Ni, Ba < 0.14 ppm; Al = 1.1 ppm) were found,.

Spray drying

For the spray drying experiments, 2.5 gram of lyophilized cetrorelix was dissolved in a mixture of 40 ml tert-butanol (TBA) and 60 ml of distilled

water. Tert-butanol was added because it was described (32) that this solvent creates improved dispersion characteristics. The resulting solution was then spray dried with a Büchi 190 Mini Spray Dryer (Büchi, Flawil, Switzerland) equipped with a two-fluid nozzle (0.5 mm). The setting of the pump was 3 (corresponding to 2.37 ml/min), aspirator 18 (-75 mbar), heating 8.5 and the atomizing airflow was 800 l_n/h. These settings resulted in an in- and outlet temperature of 117 and 75 °C, respectively. The obtained powder was subsequently stored at 20 °C and 50% RH.

Spray freeze drying
For spray freeze drying, the same solution of 2.5 gram cetrorelix in a 40/60 TBA/water (v/v) mixture was used. The solution was sprayed into a bowl of liquid nitrogen with the two-fluid nozzle (diameter 0.5 mm) of the Büchi Mini Spray Dryer. The nozzle was placed approximately 10 cm above the surface of the liquid nitrogen and the cetrorelix-solution was atomized with an air flow of 800 l_n/h. After the spraying was completed, the bowl with liquid nitrogen and cetrorelix was transferred to a Christ model Alpha 2-4 freeze dryer (Salm & Kipp, Breukelen, The Netherlands) and subsequently freeze dried at a shelf temperature of -35 °C and a pressure of 0.220 mbar. Secondary drying was started after 21 h. by gradually increasing the shelf temperature to 20 °C and decreasing the pressure to 0.060 mbar over a time period of 3 h. Secondary drying lasted another 24 hours. The obtained powder was subsequently stored at 20 °C and 50% RH.

Preparation of adhesive mixtures
The dose for Cetrorelix when administered subcutaneously is 0.25 mg. Based on this parenteral dose, the required fine particle dose for pulmonary administration was estimated to be around 0.30 mg. To deliver 0.30 mg as fine particle mass it was calculated that for a fine particle fraction of 40% a dose of 0.75 mg would be sufficient. When adhesive mixtures with a 5% w/w drug load are used, this would mean that the dose weight would be 15 mg of powder.
Adhesive mixtures were prepared by mixing Cetrorelix with Pharmatose 110 M (63-90 µm) in a tumbling mixer (Turbula T2C, W.A. Bachofen, Basel, Switzerland) at 90 r.p.m. for 30 min. in a stainless steel container of 40 ml. To obtain adequate coefficients of variation for milled cetrorelix it was necessary to use two stainless steel balls with a diameter

of 7 mm, as grinders for the drug, since the milled cetrorelix contained many agglomerates. The mixtures were stored for at least 7 days at 20 °C and 50% RH before use to reduce the effects of electrostatic charging.

Physical characterization of the starting materials and mixtures

Characterization of materials

The specific surface areas (m^2/g) of all materials (i.e. milled, spray dried and spray freeze dried cetrorelix and Pharmatose) were obtained by BET (nitrogen adsorption) measurement with a Tristar 3000 model (Micromeritics, Norcross, U.S.A.) using a 5 point analysis at -195.65 °C. The samples were inserted into test tubes and rinsed with helium prior to the measurement.

The apparent particle densities of the micronized materials were measured at a temperature of 20 °C with helium pycnometry (Quantachrome, Syosset, NY, USA).

Moisture isotherms of micronized cetrorelix were determined with a dynamic vapour sorption apparatus (DVS 1000, Surface Measurement Systems Ltd., London, UK) at 25°C. About 10 mg of material was weighed in the sample cup and exposed to 0% RH until the rate in change of mass per minute (dm/dt) was less than 0.0005%/min. The relative humidity was subsequently increased with incremental steps of 10% from 0 to 90% RH. Each step was triggered on a dm/dt of 0.0005%/min.

Particle size distributions of the micronized materials were measured in duplicate (differences for all samples were <1%) with a HELOS Compact model KA laser diffraction apparatus (Sympatec GmbH, Clausthal-Zellerfeld, Germany: 100 mm lens and the Fraunhofer theory) equipped with a RODOS disperser. The powders were dispersed at a pressure of 5 bar.

Scanning electron micrographs (SEM) were recorded with a JEOL JSM 6301-F Microscope (JEOL, Japan). The powder was dispersed on top of double-sided sticky carbon tape on metal disks and coated with 150 nm of gold/palladium in a Balzers 120B sputtering device (Balzers UNION, Liechtenstein).

The purity of the starting materials, as well as that of the raw material of cetrorelix was determined with HPLC (33). The elution condition for the peptide from the column (Lichrospher 60 RP-Select B 5 microns, 250x4 mm I.D.) was isocratic with a 49:51 mixture of an ammonium acetate buffer (0.05 mol/l, pH 4) and methanol-acetonitrile (1:1 v/v),

respectively. The detection of the peptide was done with fluorescence detection with an excitation wavelength of 227 nm and emission wavelength of 340 nm.

Characterization and dosing of mixtures
The adhesive mixtures were filled out in the dosing system of the inhalers. For the Novolizer cartridges were filled and dosing was performed with the built-in metering system, while for the ISF device, capsules were filled to the same degree as the commercial product which corresponded to approximately 25 mg. The consistency of the delivered dose from the Novolizer (Sofotec, Frankfurt, Germany) was determined by dosing 20 samples which were individually weighed. Each dose was dissolved in 25 ml de-ionized water and analyzed spectrophotometrically at 226 nm with a Philips PU 8720 UV-VIS spectrophotometer (Philips, The Netherlands). Results of dose consistency were also used for the mixtures used in the ISF inhaler.

Aerosol characterization
Particle size distributions of aerosols generated by formulations of milled, spray dried and spray freeze dried cetrorelix, respectively, were measured with a HELOS Compact model KA laser diffraction apparatus (100 mm lens and the Fraunhofer theory) equipped with the INHALER adapter (Sympatec GmbH, Clausthal-Zellerfeld) (34, 35). The powders were dispersed with the Novolizer at a pressure-difference of 4 kPa (71 l/min) across the inhaler for 3 s. The obtained data often show a bimodal size distribution in which the first peak is that of the micronized drug and the distinct, second peak is from the carrier. To obtain an estimate of the drug size distribution, the second distinct peak of the coarser carrier material was eliminated from the curve. This procedure has been validated by checking whether the second peak (coarse carrier) in the original bimodal size distribution really was originating from the carrier material. This was done by removing the particle size distribution of the drug (the first peak) and subsequent comparison of the of the obtained carrier particle size distribution with the original carrier particle size distribution. If discrepancies in the carrier size distributions were found, then the measurement was considered as wrong and a new measurement was performed.

Cascade impactor analysis was carried out with a glass constructed four stage liquid impactor of the Fisons type (Elgebe, Leek, The Netherlands). The ISF inhaler or the Novolizer was attached to a dry bent induction port. The airflow, created with a pressure-difference of 2.8 kPa (90 l/min, theoretical cut-off diameters 13.32, 6.77 and 3.35 µm for the first, second and third stage, respectively) for the ISF inhaler or 4 kPa (71 l/min, theoretical cut-off diameters 14.80, 7.58 and 3.74 µm for the first, second and third stage, respectively) for the Novolizer through the inhaler and cascade impactor was controlled with a solenoid valve in combination with a time controller to start and stop the flow. The pressure-difference of 4 kPa for the ISF inhaler could not be reached due to the low resistance of this inhaler and disruption of the capsule. The European Pharmacopoeia (2002) describes that for capsule inhalers a flow rate of 100 l/min should be used. However, due to the relative high resistance of the Fisons cascade impactor only 90 l/min was achievable which corresponds to 2.8 kPa. For each cascade impactor analysis a total dose of 125 mg had to be delivered. For the Novolizer the total mass was delivered in 10 doses, while for the ISF inhaler this was achieved in 5 doses. The difference in the number of doses was due to the fact that the capsules of the ISF inhaler had to be filled to the same degree as the marketed product in order to utilize the ISF inhaler to the best performance. Cascade impactor measurements were performed in duplicate. The amount of drug deposited on the different stages was determined by dissolving in water followed by ultra-violet spectroscopy (226 nm). Depositions on the stages are expressed as a percentage of the metered dose. The fine particle fraction is the sum of the deposition on the third and fourth stage.

RESULTS

Effect of preparation technique on cetrorelix properties

Table 1 shows that all three micronization techniques could be applied without degradation of cetrorelix. This shows that a dry powder formulation of cetrorelix can be obtained without complex formulation methods and that we can use dry powder formulation techniques based on the pure micronized drug. With respect to crystallinity it should be mentioned that all three different cetrorelix materials were amorphous, as was confirmed by differential scanning calorimetry (data not shown).

Table 1. Physical characteristics of the starting materials

Material	Type	Purity[1] (%)	Surface Area (m^2/g)	Density (g/cm^3)
Pharmatose 110M	Size fraction 63-90 µm	-	0.171	-
Cetrorelix	Milled	103.8	20.09	1.276
	Spray dried	100.6	9.15	1.187
	Spray freeze dried	98.3	49.23	0.675

[1] The purity is defined as the ratio between the amount of active before and after the micronization step multiplied by 100%

Table 2. Details of micronized cetrorelix as starting materials and in the aerosol from the Novolizer

		x_{10} (µm)	x_{50} (µm)	x_{90} (µm)
Milled Cetrorelix	Starting material	0.55	1.34	48.50
	Aerosol cloud	0.56	1.38	3.11
Spray dried Cetrorelix	Starting material	1.02	2.30	4.62
	Aerosol cloud	1.42	2.58	4.52
Spray freeze dried Cetrorelix	Starting material	0.90	2.32	5.22
	Aerosol cloud	0.78	2.13	6.73

In Fig. 1 the particle size distributions of the pure micronized materials from RODOS dispersion are shown, and in Table 2 the accompanying 10%, 50% and 90% values from the cumulative undersize curves are presented. As can be seen from the bimodal shape of the cumulative curve, milled cetrorelix contains several agglomerates larger than 10 µm in the starting material. Fig. 1 also shows that spray dried and spray freeze dried cetrorelix have almost similar median particle size diameters. Clearly all three micronization techniques result in particles that are within the respirable range.

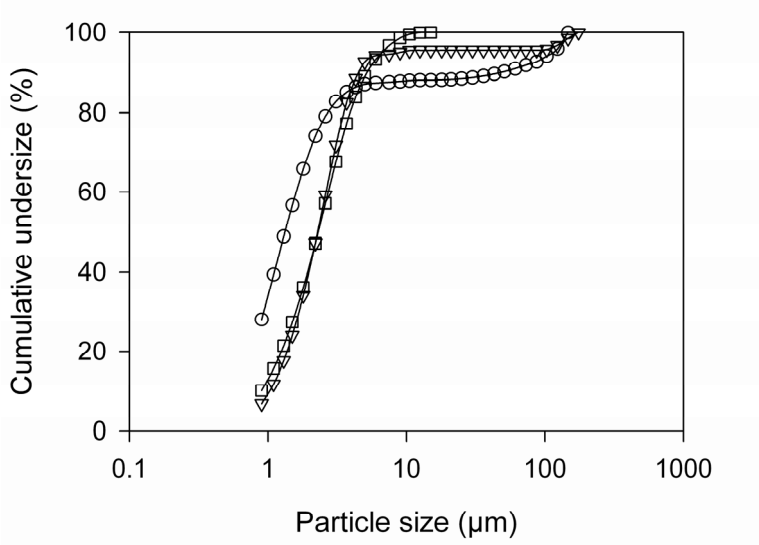

Figure 1. Particle size distributions of micronized cetrorelix. Open symbols represent the particle size distributions of the starting materials obtained from RODOS dispersion (○ = milled cetrorelix, ▽ = spray dried and □ = spray freeze dried).

The densities of milled and spray dried cetrorelix (Table 1) are similar. The spray freeze dried powder has a significantly lower density than the two other products; indicating a higher porosity of this material.
The specific surface area of the micronized materials differ considerably (Table 1). Spray dried cetrorelix has the lowest specific surface area, whereas milled and especially spray freeze dried cetrorelix have larger surface areas. Scanning electron micrographs (SEM's, Fig. 2) reveal that milled cetrorelix (Fig. 2a) consists of agglomerates of very small primary particles which are mostly smaller than 1 µm. Spray dried cetrorelix has a different – flake like - shape (Fig. 2b) and a larger particle size compared to milled cetrorelix. Spray freeze dried cetrorelix (Fig. 2c) seems to be much more porous than spray dried cetrorelix although the particle size is similar.

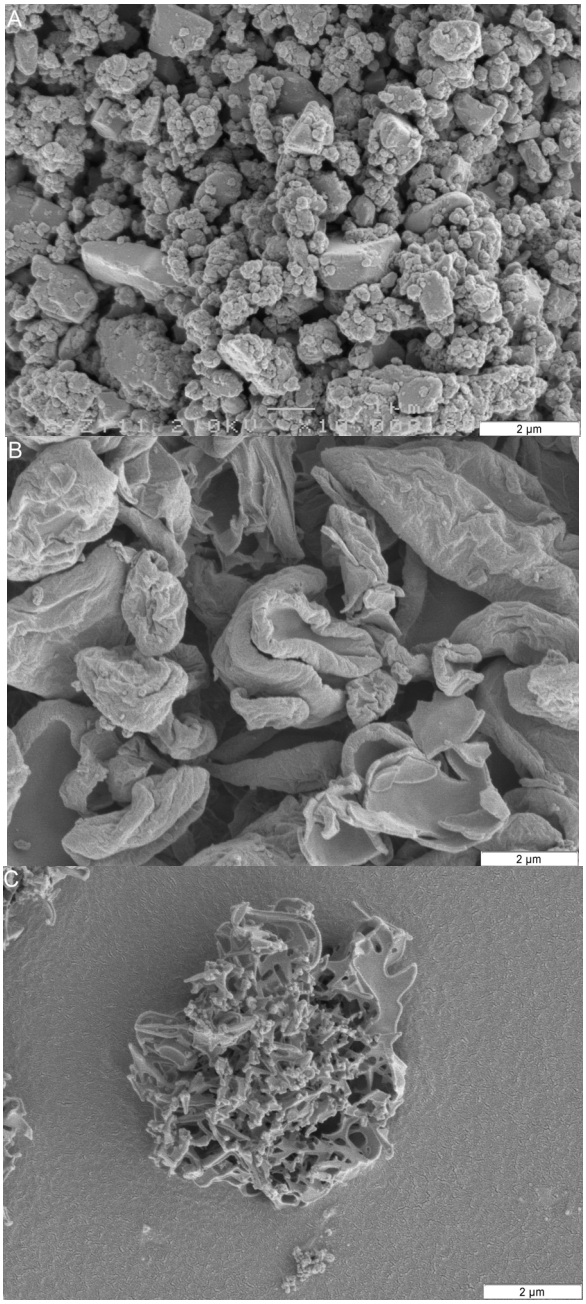

Figure 2. Scanning electron micrographs of micronized cetrorelix by milling (a,), spray drying (b) and spray freeze drying (c). The photographic magnification in all three micrographs is 10,000 times.

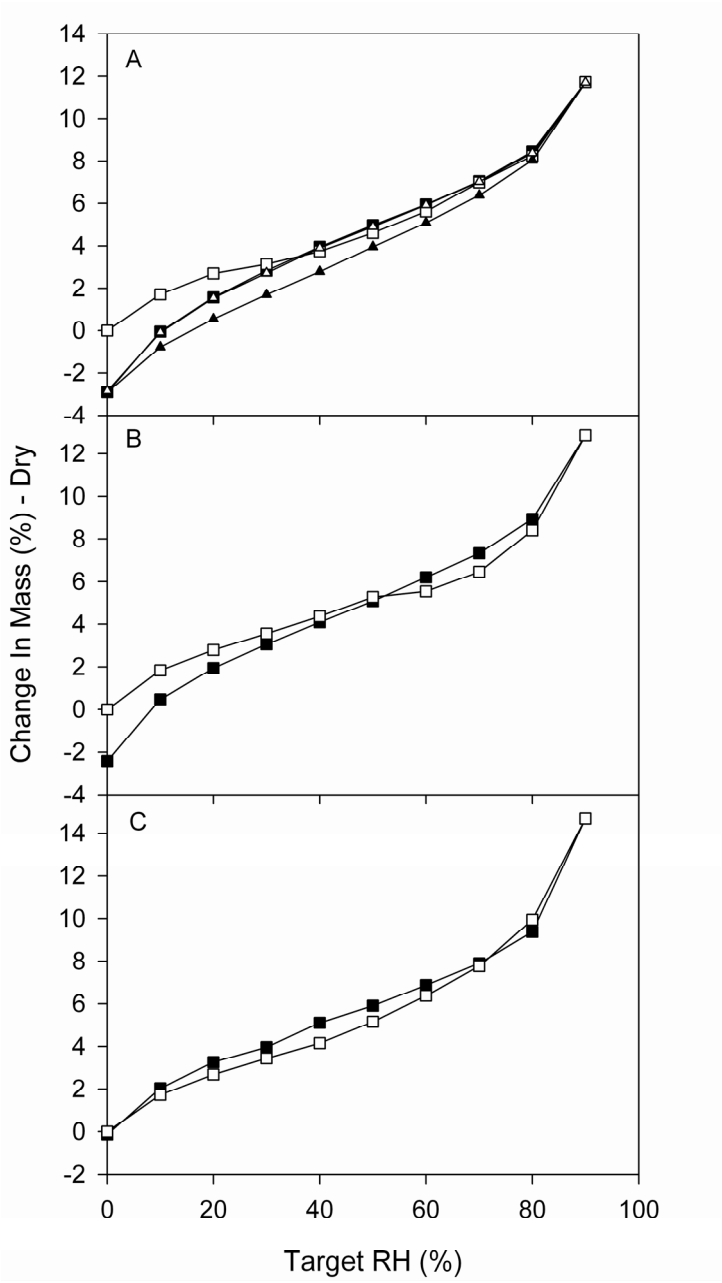

Figure 3. Dynamic vapour sorption studies of micronized cetrorelix (a), spray dried cetrorelix (b) and spray freeze dried cetrorelix (c). Open symbols represent vapour sorption, closed symbols vapour desorption. Squares represent the first measurement, triangles the second measurement.

Results of the dynamic vapour sorption studies are given in Fig. 3. Fig. 3a shows the results of two consecutive DVS measurements of milled cetrorelix. It can be seen that the difference in mass after the first sorption and desorption cycle is about 3%. After the second cycle, no significant change in mass can be observed. Spray dried cetrorelix, which is shown in Fig. 3b, shows the same trend as the first cycle of milled cetrorelix. Finally, spray freeze dried cetrorelix (Fig. 3c) shows no change in mass at all between the start and end of the sorption cycle. This is similar to the second sorption and desorption cycle of the milled cetrorelix. Obviously, the exposure to very low pressure (freeze drying) changes the behaviour during DVS measurements. This change also occurred after a full sorption-desorption cycle. This shows that although a minor volatile impurity is removed from the milled and spray dried material during the first cycle, no significant differences occur between the surface properties of the three different materials.

Physical properties of the adhesive mixtures

Mixing experiments with pure carrier material with and without stainless steel balls indicated that the presence of these balls has no significant effect on the particle size and morphology of the carrier (results not shown).

Table 3 shows the characteristics of all produced adhesive mixtures. We produced three adhesive mixtures with 5% (w/w) carrier payload (Pharmatose as carrier). The mixture with spray dried cetrorelix had the highest coefficient of variance (CV= 4.28%), the lowest dosing weight and the highest spread in the dosing weight. However, all formulations met the requirement of a coefficient of variance below 5%.

Table 3. Overview of the used adhesive mixtures and dose metering from the Novolizer

Carrier	Drug	Drugload (w/w drug/carrier)	CV	Dosing weight (mg ± SD)
Pharmatose	Milled cetrorelix	4.73%	1.95%	13.19 ± 0.33
Pharmatose	Spray dried cetrorelix	4.49%	4.28%	12.56 ± 1.07
Pharmatose	Spray freeze dried cetrorelix	4.72%	1.65%	13.02 ± 0.26

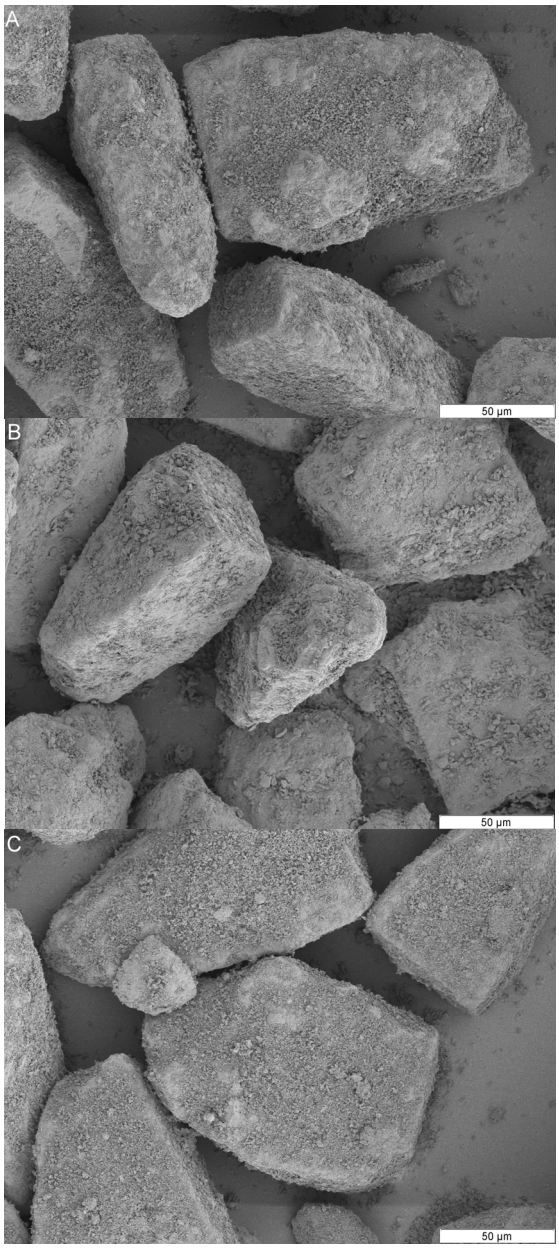

Figure 4. Scanning electron micrographs of the adhesive mixtures at a magnification of 500x of milled cetrorelix/pharmatose (a), spray dried cetrorelix (b) and spray freeze dried cetrorelix (c).

Scanning electron micrographs of the micronized materials formulated as an adhesive mixture with Pharmatose (5% w/w) are shown in Fig. 4. Milled cetrorelix (Fig. 4a) and spray dried cetrorelix (Fig. 4b) appear to spread over the surface of the carrier with a tendency to be stowed away in irregularities. The micrographs also reveal that the spray freeze dried material (Fig. 4c) has a smaller particle size after formulation into an adhesive mixture compared to the starting material.

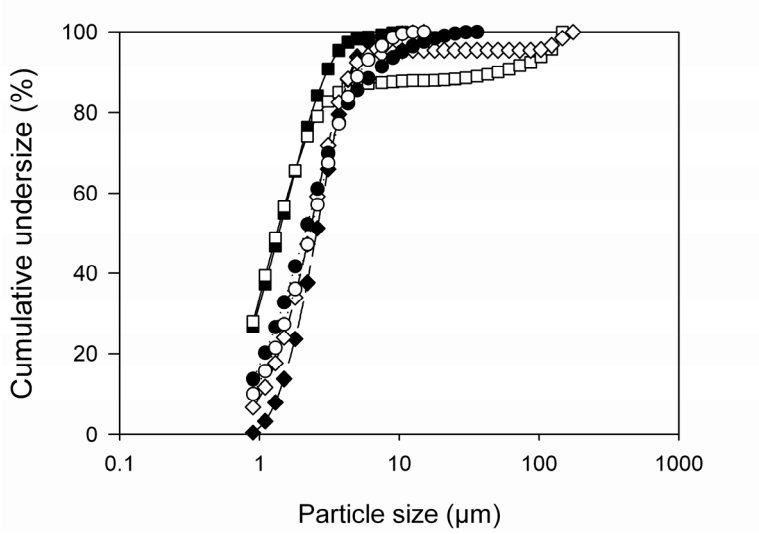

Figure 5. Particle size distributions of cetrorelix. Open symbols represent the particle size distributions of the starting materials obtained from RODOS dispersion, closed symbols represent the particle size distributions of the emitted aerosol-cloud from the Novolizer for the 5% w/w adhesive mixtures (■ & solid line = milled cetrorelix, ♦ & dashed line = spray dried and ● & dotted line = spray freeze dried).

Effect of the preparation technique and device on aerosol properties of cetrorelix

Fig. 5 shows the effect of formulation on the particle size distribution in the aerosol cloud from the Novolizer compared to the particle size distribution of the starting materials obtained from RODOS dispersion. The cumulative undersize values of the data presented in Fig. 5 are depicted in Table 2. The size distributions the starting material and formulated drug are very similar for milled cetrorelix. Only the x_{90}-value for the starting material is higher; this is explained by the presence of

agglomerates as described above. These agglomerates are not present in the formulation, showing that the stainless steel balls in the mixing process were effective in breaking up agglomerates. For the other two materials the size distributions of starting material and formulated drug were similar. However, the fine particle fraction obtained by cascade impactor analysis with the Novolizer with this spray freeze dried material was only 25 %. This was considered insufficient and no further experiments were carried out with this material.

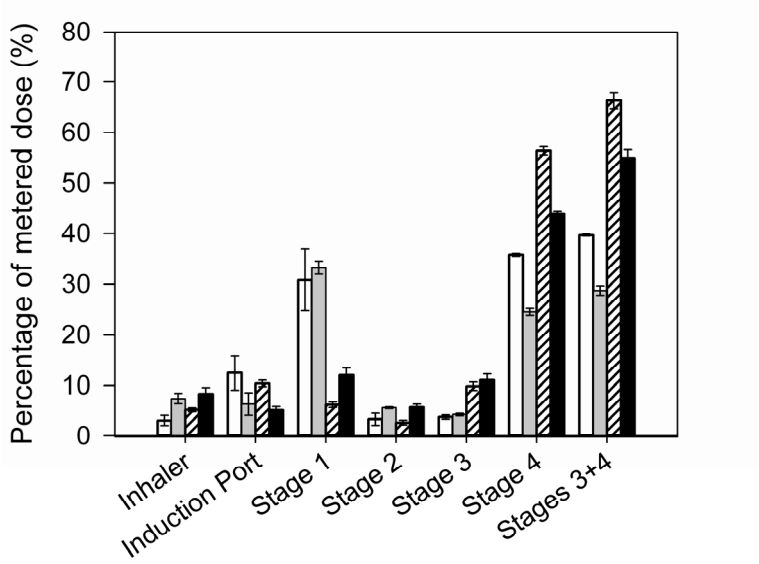

Figure 6. Results from cascade impactor analysis of 5% w/w adhesive mixtures of micronized cetrorelix by milling and spray drying at 2.8 kPa with the ISF inhaler and 4 kPa with the Novolizer. The white and grey bar represent dispersions of milled cetrorelix dispersed with the ISF inhaler (white bars) and Novolizer (grey bars), whereas the patterned and black bars represent dispersions of spray dried cetrorelix with the ISF inhaler (patterned bars) and Novolizer (black bars). Error bars represent the highest and lowest value.

Fig. 6 shows the results from cascade impactor analysis for the 5% mixtures with milled and spray dried cetrorelix, respectively as performed with the ISF inhaler and the Novolizer. With the ISF inhaler, device accumulation is higher than for the Novolizer, but loss in the induction port is higher for the Novolizer. This is due to centrifugal exit of the aerosol cloud from the mouthpiece. Furthermore, for milled

cetrorelix the first stage deposition is relatively high with either the ISF inhaler or Novolizer. First stage deposition is much smaller for spray dried cetrorelix, also resulting in higher fine particle fractions (Stages 3 + 4). The differences between both the type of micronization and the type of inhaler is remarkable.
We performed univariate analysis of variance on the obtained fine particles fractions. We observed large differences between both the type of inhaler ($p= 0.001$, 95% confidence interval: 7.97 – 14.24) and method of micronization ($p<0.001$, 95% confidence interval: 23.26 – 29.53).

DISCUSSION

Advanced formulation techniques, like the use of Force Control Agents or large porous particles may be necessary to obtain the required quality attributes for an inhaler product. Typical examples are stabilization of unstable proteins (36, 37), improvement of de-agglomeration (into an inhalable aerosol) and improvement of flow properties (necessary for processing of the bulk powder). However, whether all these technologies always need to be applied depends on the complex interaction between parameters such as the requirements stemming from the nature of the drug product, the type of formulation used and the de-agglomeration efficiency of the inhaler. For cetrorelix we showed that the different micronization techniques could be applied without further degradation of the material. The different micronization techniques resulted in particles with different physical characteristics which determined their performance when formulated in an adhesive mixture.
The milling process used in this study resulted in agglomerates consisting of primary particles which were generally smaller than 1 µm, but larger agglomerates of more than 10 µm were also found. This necessitated the use of grinders, such as stainless steel balls, during mixing. However, as a result of this mixing process the (small) drug particles were firmly pressed on the carrier which increased the adhesive forces; this had a negative effect on the FPF obtained with this formulation.
The observation that spray dried Cetrorelix had a higher fine particle fraction than milled Cetrorelix is explained both by the particle size of the drug powder and the (flake like) shape of this powder. As a result of the irregular surface of spray dried cetrorelix, the de-agglomeration into an aerosol is improved, as shown by the low first stage deposition (Fig. 6).

Finally the improved aerosol characteristics (aerodynamic diameter) of this product should be mentioned. Based on the equation (14, 38):

$$d_{ae} = d_e \sqrt{\frac{\rho_p}{\rho_0 \chi}},$$

which is only valid in the Stokes regime, a larger aerodynamic shape factor (x) of spray dried cetrorelix (flakes) results in a lower aerodynamic diameter (d_{ae}) compared to milled cetrorelix (spheres) which has a profound effect on the deposition of the particles as measured by cascade impactor analysis. This is explained by the fact that particles with a high aerodynamic shape factor (higher resistance) can accelerate very quickly in a changing air flow (change of particle trajectory). In fact, Shekunov et al. (23) found comparable results upon comparison of milled and supercritically-processed salmeterol xinafoate, the higher aerodynamic shape factor of the later material, improved dispersion characteristics and yielded a higher FPF.

The physical characteristics we found for the spray freeze dried product are comparable to those found by other authors, e.g. large surface area, high porosity (21, 39). However, the performance of the spray freeze dried powder in the adhesive mixture was poorest. This is ascribed to the fact that this advanced particle engineering technique results in very fragile particles which cannot withstand the production process of an adhesive mixture. During the mixing process, the fragile particles of spray freeze dried cetrorelix are exposed to impact forces from colliding lactose particles. These forces crush the fragile spray freeze dried cetrorelix particles into smaller fragments. Once attached to the carrier, these very small fragments are more difficult to separate again from the carrier during inhalation because of their small diameter and low density (small amount of mass per particle). This results in relatively low separation (impaction) forces, since the force resulting from an impaction is depending on mass ($F = m \cdot a$). The low separation force explains the low FPF during cascade impactor analysis. This shows that the type of formulation largely influences the performance of particles processed with different techniques. Obviously, spray freeze drying produces large porous particles that are not suitable to be processed into an adhesive mixture. On the other hand, large porous particles were successfully applied in other types of formulations (27, 40, 41). Therefore, formulation types and processes that could be survived by large porous

particles would still be of interest since they would potentially allow the advantages in de-agglomeration and aerodynamic behaviour of this particle type.
Next to the physical characteristics of the drug particles in the adhesive mixtures the fine particle fraction generated by a dry powder inhalation system is also determined by the efficiency of the de-agglomeration principle applied in the inhaler. The higher de-agglomeration efficiency of the Novolizer is reflected in the lower first stage deposition compared to the ISF inhaler. On the first stage, mainly drug particles that are still attached to the carrier are deposited. For both formulations the first stage drug deposition is higher for the ISF inhaler.
It is clear that the higher de-agglomeration efficiency of the air classifier in the Novolizer results in higher fine particle fractions than the fractions obtained with the ISF inhaler. Fig. 6 furthermore shows that the magnitude of the difference between the de-agglomeration principles was independent from the different drug particle types (milled vs. spray dried) used in the formulations. For both types of formulations an increase of about 11% in FPF was found. The difference in FPF caused by the change in particle type was for both inhalers about 26% whereas the more efficient de-agglomeration principle yielded an increase of only 11% for both formulations. Shekunov et al. (23) stated that powders with low dispersion forces (e.g. spray dried cetrorelix) would exhibit device independent performance. However, our results show that the performance in terms of fine particle fraction is actually dependent on the device, although the role of particle physics is more important in the systems tested in this study.
Finally, it should be mentioned that the lower first stage deposition of the Novolizer would not only reduce the waste of valuable drug but could also potentially reduce side effects.

CONCLUSIONS

The effects of different techniques for micronization used in the development of a peptide-containing dry powder formulation for inhalation were investigated. High fine particle fractions in the aerosol are required and losses during inhalation should be minimized to obtain the highest possible effect and lowest waste of drug. We showed that it is possible for a stable peptide to be micronized with milling, spray drying and spray freeze drying without degradation of the drug. Whether a

micronization technique produces particles that are suitable for use in an inhalation formulation was found to depend on the type of formulation and formulation process used. Furthermore, the performance of the different formulations (aerosolization behaviour expressed as fine particle fraction) was found to depend on the efficiency of the de-agglomeration principle applied.
By combining the best particle engineering technique for this type of formulations with the most efficient de-agglomeration principle very high 3rd and 4th stage depositions could be obtained. Such an inhalation system could be further developed to be a non-invasive alternative for routine administration of cetrorelix.

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CHAPTER 3

Characterization of a cyclosporine A solid dispersion for inhalation

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ABSTRACT

For lung transplant patients, a respirable, inulin-based solid dispersion containing cyclosporine A (CsA) has been developed. The solid dispersions were prepared by spray-freeze drying. The solid dispersion was characterized by water vapor uptake, specific surface area analysis and particle size analysis. Furthermore, the mode of inclusion of CsA in the dispersion was investigated with Fourier transform infrared spectroscopy. Finally, the dissolution behavior was determined and the aerosol, that was formed by the powder, was characterized. The powder had large specific surface areas (~ 160 m^2). The water vapor uptake was dependant linearly on the drug load. The type of solid dispersion was a combination of a solid solution and solid suspension. At a 10% drug load, 55% of the CsA in the powder was in the form of a solid solution and 45% as solid suspension. At 50% drug load, the powder contained 90% of CsA as solid suspension. The powder showed excellent dispersion characteristics as shown by the high emitted fraction (95%), respirable fraction (75%) and fine particle fraction (50%). The solid dispersions consisted of relatively large ($x_{50} \approx$ 7 μm), but low density particles ($\rho \approx 0.2$ g/cm^3). The solid dispersions dissolved faster than the physical mixture, and inulin dissolved faster than CsA. The spray freeze drying with inulin increased the specific surface area and wettability of CsA. In conclusion, the developed powder seems suitable for inhalation in the local treatment of lung transplant patients.

INTRODUCTION

To improve the immunosuppressive therapy in lung transplant patients, studies with inhalation of cyclosporine A (CsA, Figure 1) have been conducted (1, 2). The overall survival of lung transplant patients increased dramatically when aerosolized CsA was administered on top of base therapy (3). The formulation for inhalation used in the published study was a solution of CsA in propylene glycol. A disadvantage of this approach was that nebulization of propylene glycol caused extreme irritation in the airways, which was reduced by the use of aerosolized local anesthetics. Despite the use of local anesthesia, a substantial number of patients (± 10%) could not complete the study due to extreme irritation (4). Apparently, there is a need for a less irritating formulation for inhalation. A second disadvantage of nebulization of CsA dissolved in propylene glycol is the low deposition efficiency, which has been reported to be 5.4 - 11.2% of the metered dose (4), which is consistent with other studies on nebulization (5-7). To obtain a significant improvement in pulmonary function in lung transplant patients with chronic rejection requires a deposition of at least 5 mg CsA in the lower airways (peripheral part of the lung) (1), which can only be achieved by high metered doses. This further underlines the need for more effective formulations for inhalation. A third problem is the lipophilicity of CsA (aqueous solubility of 3.69 mg/L at 37 °C, log P = 3.0) (8, 9), which limits the choice of solvents for nebulization.

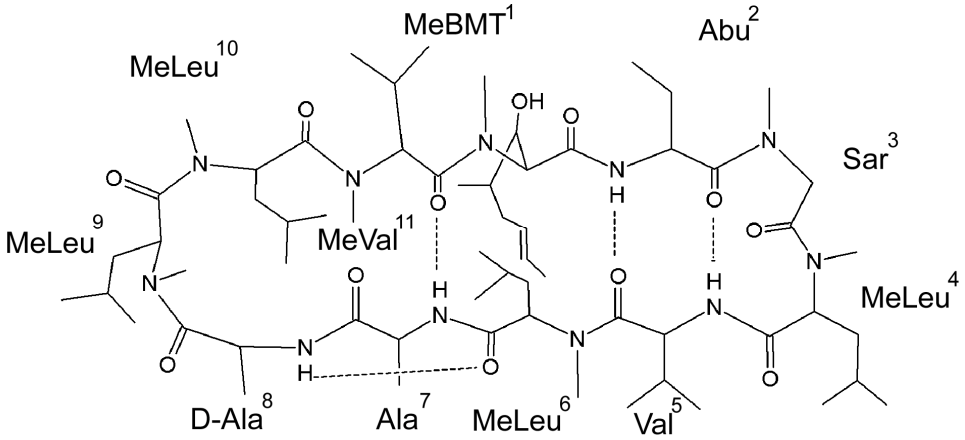


Figure 1. Molecular structure of Cyclosporine A.

We proposed that a dry powder formulation consisting of a solid dispersion could overcome these problems. Firstly, with dry powder inhalation the use of local anesthesia and irritating solvents can be avoided. Secondly, the higher deposition efficiency of dry powder inhalation compared to nebulization reduces the need for high metered doses (10). Finally, solid dispersions are known for their dissolution rate enhancing properties of poorly soluble drugs, such as CsA (11-13). The dissolution rate of poorly soluble drugs from inulin-based solid dispersions has been shown to increase when compared to the pure hydrophobic drug (14).

A powder for local administration in lung transplant patients should meet two requirements. Firstly, the drug substance should dissolve within a reasonable time span, since removal of particles by mucociliary clearance occurs within a couple of hours (15, 16). Secondly, the powder should have an aerodynamic size range which is suitable for inhalation (1 – 5 μm) (17, 18). Regarding the first requirement, benefit may be gained with a rapidly dissolving dry powder formulation. The second requirement can be met by using spray freeze drying as production method (19-21).

The purpose of this study was to develop a powder for inhalation as an alternative for nebulized CsA. We investigated the feasibility of an inulin-based solid dispersion with CsA. CsA in the solid dispersion can be distributed as separate molecules, defined as a solid solution, or as multimolecular clusters of CsA, defined as a solid suspension or as combination of both (22). The type of solid dispersion of CsA in inulin, e.g. solid solution, solid suspension or a combination of both was investigated with Fourier transform infrared spectroscopy. Furthermore, other physical characteristics relevant for the performance as powder for inhalation were investigated.

MATERIALS AND METHODS

Materials

CsA was obtained from LC Labs (Woburn, MA, USA). Inulin, with an average degree op polymerization of 23 was a generous gift from Sensus (Roosendaal, The Netherlands). *Tert*-butyl alcohol (TBA), methanol, acetonitrile, sodium dodecyl sulphate (SDS), KBr and anthrone reagent were of the highest available grade of purity (KBr was spectroscopy grade) and purchased from commercial suppliers. The water used in the experiments was demineralized.

Methods

Sample preparation

The CsA solid dispersions were produced as described previously (20). Briefly, CsA was dissolved in TBA and inulin in water. Next, the solutions were mixed at a TBA/water ratio of 40/60 v/v. The concentrations of CsA in TBA and inulin in water were chosen such that the combined mass of CsA and inulin in the solution was constant (5% w/v) while the drug load varied. Immediately after mixing, the resulting TBA/water solution was sprayed onto liquid nitrogen with a heated two-fluid nozzle (orifice 0.5 mm, atomizing airflow 500 L/h, liquid flow 6 mL/min). The solutions did not show any phase separation as indicated by clearness of the solution during processing. Upon completion of spraying the liquid nitrogen was evaporated and the frozen droplets were freeze dried in a Christ model Alpha 2-4 lyophilizer (Salm and Kipp, Breukelen, The Netherlands). The shelf temperature of the lyophilizer was maintained at -35 ºC with a pressure of 0.220 mbar for 24 hours. The temperature was then gradually increased to 20 ºC, while the pressure was decreased to 0.050 mbar. These conditions were maintained for another 24 hours. During the whole freeze drying process the condenser temperature remained -55 ºC. After freeze drying the samples were stored over silica gel in a vacuum desiccator until analysis.

Chemical analysis

The drug content and concentration during dissolution was determined by an HPLC method(23) which was slightly modified for our purpose. In short, undiluted samples (100 µL) were injected using a Gilson 234 auto injector (Gilson, Middleton, WI, USA) on a reversed phase C8 column (5 µm Hypersil BDS 250 x 4.6 mm, Thermo Hypersil Ltd., Runcorn, UK) equipped with a thermostat (Jones Chromatography model 7990) at 70 ºC. A flow rate of 0.9 mL/min of the mobile phase (85/15 acetonitrile/water) was maintained with a Waters model 510 pump (Waters, Milford, MA, USA). Detection of CsA was performed with a Waters 490 programmable wavelength detector (Waters, Milford, MA, USA) at 212 nm. These settings resulted in an elution time for CsA of approximately 6 minutes. The obtained data was analyzed with KromaSystem 2000 software version 1.83 (Winooski, VT, USA). All measurements were performed in triplicate. Drug content was within ±2.5% of the theoretical drug content and dissolution of CsA was 100±2.5% of the metered dose for dissolution.

The concentration of inulin during dissolution was determined by the anthrone assay (24). Samples of 0.25 mL were diluted with water to 1.00 mL. Subsequently, 2.00 mL of a freshly prepared 0.1% w/v anthrone reagent in concentrated sulfuric acid was added and immediately vortexed. Due to the exothermic character of the reaction enthalpy of mixing, the mixture heated up. After cooling to room temperature samples (200 µL) were pipetted in a 96-wells plate and analyzed spectrophotometrically at 630 nm in a plate reader (Benchmark plate reader, BioRad, Hercules, USA). A freshly prepared calibration curve of inulin (0 – 0.100 mg/mL, 11 points) was measured for each determination. All measurements were performed in triplicate.

Physical characterization of samples

Specific surface areas of the formulations were measured using the Brunauer, Emmett and Teller (BET) method with a Tristar 3000 model (Micromeritics, Norcross, USA). Prior to the measurement samples were placed into test tubes and purged for at least 3 hours with helium at 30 ºC to evaporate residual moisture. Subsequently, a nine point isotherm was recorded at a sample temperature of -196 ºC (liquid nitrogen).

The vapor sorption behavior of the solid dispersions at 25 °C was determined by dynamic vapor sorption (DVS 1000, Surface Measurement Systems Ltd., London, UK). About 10 mg of material was weighed in the sample cup and exposed to 0% relative humidity (RH) until equilibrium (dm/dt<0.0005%/min) was reached. The RH was subsequently increased to 30%. An RH of 30% was chosen because inulin remains in the glassy state upon exposure to 30% RH at 25 °C (9, 25).

Changes in the secondary structure of CsA caused by spray freeze drying and incorporation in inulin were studied with Fourier transform infrared (FTIR) spectroscopy. Powder samples were prepared by mixing ~ 2-3 mg of the different CsA formulations with ~ 300 mg KBr. These mixtures were compacted into a 13 mm disc at 37 MPa pressure with a hydraulic press. The liquid sample was prepared by dissolving CsA in a mixture of methanol and acetonitrile. Water was gently added to obtain a concentration of 100 mg/mL and volume percentages of 32% MeOH, 32% ACN and 36% H_2O. Spectra of dry powders were recorded on a Bio-Rad FTS6000 FTIR spectrometer with Win-IR Pro software (Cambridge, MA, USA). 256 Scans were co-added at a resolution of 2 cm^{-1} and scan speed of 0.16 cm/s (5 kHz laser modulation). Spectra of CsA in the co-solvent

solution were collected by averaging 1024 scans. All absorbance spectra were corrected for water vapor and if necessary for the co-solvent solution (26). The measured spectra were smoothed with a 13-point Savitzky-Golay function (27) and second derivative spectra were calculated. The derivative spectra were also smoothed with a 13-point Savitzky-Golay function and inverted.
To establish similarity of the unresolved amide I bands, the specific area overlap was determined (28, 29). The spectra were truncated to contain only the amide I band. Next, the amide I band was baseline-corrected, area-normalized to unity, and exported to a spreadsheet-program. The area overlap of two spectra, with the amide I band of CsA in the co-solvent solution as reference, was calculated by creation of a new spectrum consisting of the lowest absorption value of the two spectra at each data point. The area under this new spectrum corresponds to the percentage area overlap.

Aerosol characterization
Particle size distributions of the spray freeze dried samples were measured with a Helos Compact model KA laser diffraction apparatus (100 mm lens, Fraunhofer theory) equipped with a RODOS dispersing system (Sympatec GmbH, Clausthal-Zellerfeld, Germany) at a pressure difference of 0.5 bar. All measurements were performed in triplicate.
Since the particle size distributions were found to be log-normal, the geometric standard deviation (GSD) was calculated from laser diffraction data as follows (30):

$$GSD = \sqrt{\frac{x_{84}}{x_{16}}} \tag{1}$$

in which x_{84} and x_{16} represent the 84% and 16% cumulative undersize diameter, respectively. The aerodynamic diameter, as measured with cascade impactor analysis, depends on the equivalent particle diameter, shape factor and density (30):

$$d_{ae} = d_e \sqrt{\frac{\rho_p}{\rho_0 \chi}} \tag{2}$$

in which d_{ae} is the aerodynamic diameter, d_e the equivalent laser diffraction diameter, ρ_p the density of the particles (g/cm^3), ρ_0 the unit

density (1 g/cm^3) and x the dynamic shape factor (1 for spherical particles).
For cascade impactor analysis a multi-stage liquid impinger (MSLI, Erweka, Heusenstamm, Germany) was used with a test inhaler(31) attached to the induction port. A solenoid valve was used in combination with a timer to control the flow (60 L/min) through the inhaler and the cascade impactor for the duration of 3 seconds. The stages of the cascade impactor were filled with 20 mL of 85/15 v/v acetonitrile/water for extraction of CsA from the deposited powder. A total amount of 50 mg was delivered in 10 separate doses. The deposition of the drug on the different stages was measured by HPLC as described above. The deposition was subsequently calculated as the percentage of the metered dose. The emitted fraction was defined as the metered dose minus inhaler retention, the respirable fraction was calculated by the sum of deposition on 2nd, 3rd, 4th and filter stage and the fine particle fraction (FPF) was calculated by the sum of deposition on the 3rd, 4th and filter stage. The emitted fraction, respirable fraction and FPF are expressed as a percentage of the metered dose. Cascade impactor analysis was performed on only the solid dispersions with 30% and 50% w/w drug loads. All experiments were carried out in triplicate.

Dissolution rate

Dissolution experiments were carried out using an USP dissolution apparatus I (Rowa Techniek B.V., Leiderdorp, The Netherlands) at 37 °C in 1000 mL water containing 0.25% w/v SDS with constant stirring of 100 rpm. Prior to the dissolution study a series of solubility tests were conducted with SDS concentrations up to 1% w/v to investigate at which SDS concentration sink conditions (maximal concentration= $0.20 \times C_{saturated}$) were met. At the maximal CsA concentration (30 mg/L) it was found that sink conditions were met at 0.25% SDS (data not shown). The formulations were weighed into the baskets of the dissolution apparatus. For the formulations containing 10, 20, 30 and 50% w/w CsA, amounts of respectively 300, 150, 100 and 60 mg powder were weighed in order to keep the total amount of CsA in the dissolution vessel constant at 30 mg. A physical mixture of 30 mg spray freeze dried CsA and 70 mg spray freeze dried inulin was used as a reference. Samples were analyzed with HPLC and anthrone assay for determination of the CsA and inulin

concentration, respectively. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Physical characterization

In Table 1 the specific surface areas and moisture uptake of the produced solid dispersions are shown. Spray freeze drying generally results in powders with large specific surface areas and the powders in this study are no exception (20, 32, 33). The large specific surface area is related to the spray freeze drying process because with spray freeze drying the aerosol is rapidly frozen which causes the formation of a large number of small solvent crystals. Around these small solvent crystals the freeze concentrated fraction is distributed. Solvent removal by freeze drying thus results in a large specific surface area. The produced batches did not differ substantially in specific surface area, with pure spray-freeze dried CsA as an exception (Table 1). The lower specific surface area of pure spray freeze dried CsA may be caused by the absence of sugar which might influence the freezing behavior.

Table 1. Specific surface areas and water vapor uptake (at 30% RH and 25 °C) of the spray freeze dried powders.

Batch	Specific surface area (m^2/g)	Moisture uptake (% w/w)
10% w/w CsA	152	6.08
20% w/w CsA	185	5.44
30% w/w CsA	185	4.84
50% w/w CsA	145	3.97
Pure spray-freeze dried CsA	40	1.03

Possible degradation of CsA during spray freeze drying has not been investigated. However, we have no reason to assume that degradation of CsA did occur during spray freeze drying for various reasons. First of all, results from drug content analysis and dissolution were all within ±2.5% of the theoretical drug content and metered dose, respectively, which suggests a complete mass balance for CsA. Second, FTIR did not reveal possible degradation by fingerprint inspection. Furthermore, CsA appears to be a stable compound since it has been processed in several ways without noting degradation in various studies (34-36). Finally, a

chemically less stable compound than CsA, Δ^9-tetrahydrocannabinol, has successfully been spray freeze dried from a TBA/water solution without noticeable degradation (20), thus showing that spray freeze drying is a reliable production technique, which minimally distorts the formulated compounds.

The produced powders were susceptible to water vapor uptake (Table 1), which was linearly dependent on the drug ratio ([moisture content] = -0.057·[drug ratio] + 0.067; R^2= 0.995). Drug ratio was defined as [drug mass]/[drug mass + mass of excipient] without the addition of water vapor. This shows that the solid dispersion behaves like a physical mixture, which indicates that the two components remain in the same physical state, irrespective of the drug load. Furthermore, it shows that CsA does not influence the hydrophilicity of inulin and vice-versa, i.e. CsA and inulin each absorb a fixed amount of water vapor, irrespective of the drug load.

The linear dependency of moisture uptake versus drug load for inulin-based solid dispersions has been shown before (22). In that study, van Drooge et al (22) showed that the water vapor sorption of inulin-based solid dispersions was independent of the type of solid dispersion, defined as a fully amorphous solid solution, fully amorphous solid suspension or a combination of both (22). The type of solid dispersion is therefore not discernable with only water vapor sorption measurements.

In general, the physical state of solid dispersions can be determined by differential scanning calorimetry (DSC). However, DSC measurements of CsA and inulin result in substantial overlap of thermal events of both components, which makes it difficult to draw unambiguous conclusions about the physical state of the solid dispersion. Fortunately, the secondary structure of CsA, which can be measured with FTIR, has been reported to depend on the physical state (37, 38). Furthermore, FTIR might give information about the type of solid dispersion, based on the secondary structure of CsA.

CsA has four amide groups, which, according to Stevenson et al (37), in a *hydrophilic* environment result in *inter*molecularly oriented hydrogen bonds. In contrast, a *lipophilic* environment results in *intra*molecularly oriented hydrogen bonds. In the case of a solid solution, the amide groups are hypothesized to be intermolecularly oriented due to hydrogen bond interaction with inulin. Conversely, in a solid suspension, the amide groups are predominantly intramolecularly oriented due to the lipophilic

CsA clusters. A combination of a solid solution and suspension should thus result in both inter- and intramolecularly orientated amide groups, in a ratio that would be dependent on the drug load.

Secondary structure of CsA

First, two control samples, CsA in the crystalline state and dissolved in a co-solution of methanol/acetonitrile/water, were measured to show the effect of full intra- and intermolecular orientation of the amide groups, respectively. Bands were assigned according to previously published data (37).

In the crystalline state (Figure 2A), the four amide groups in the CsA molecule (Figure 1) are assumed to be intramolecularly oriented to allow intramolecular hydrogen bonds (37). In contrast, when CsA was dissolved in the co-solvent solution (Figure 2B), the amide groups are assumed to be directed outwards to allow hydrogen bonding with the solvent (37). Specifically, dissolution of CsA in the co-solvent solution compared to CsA in the crystalline state results in a β-sheet band at higher wave numbers, which is indicative of a looser β-sheet structure (Table 2). Furthermore, the γ-loop changed into a γ-turn upon dissolution in the co-solvent solution, which is associated with a change in orientation of the bond between $MeLeu^{9}$ and $MeLeu^{10}$ from cis to trans (37). This change in orientation is caused by the hydrogen bond between D-Ala^{8}NH and $MeLeu^{6}$C=O, which bifurcates to include the D-Ala^{8}C=O and solvent. The sharing of the amide proton between two carbonyls and solvent twists the backbone conformation at D-Ala^{8}, by which the orientation of the $MeLeu^{10}$ side chain changes. Finally, if the $MeBMT^{1}$ side chain (see Figure. 1) is in proximity of the backbone, as is the case in the crystalline state, then the β-OH is able to bond with $MeBMT^{1}$C=O, resulting in a MeBMT-turn (37). Clearly, the MeBMT-turn is not present upon dissolution of CsA in the co-solvent solution, which indicates that the $MeBMT^{1}$ side chain is directed outwards to interact with the solvent.

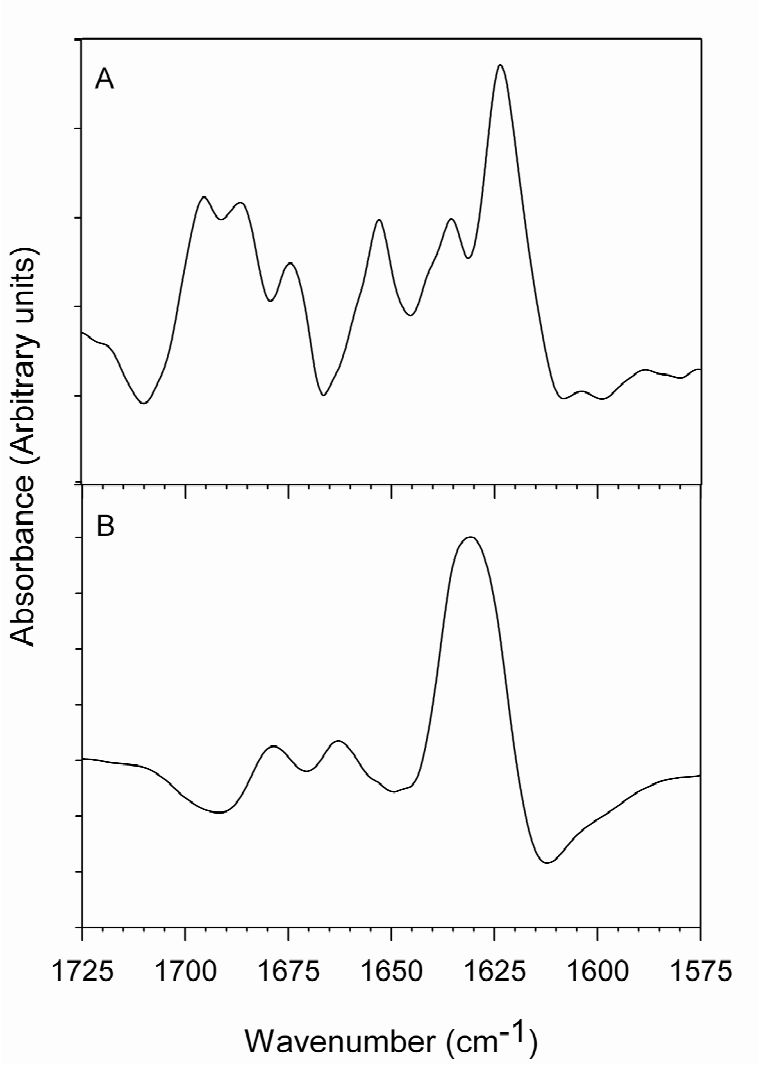
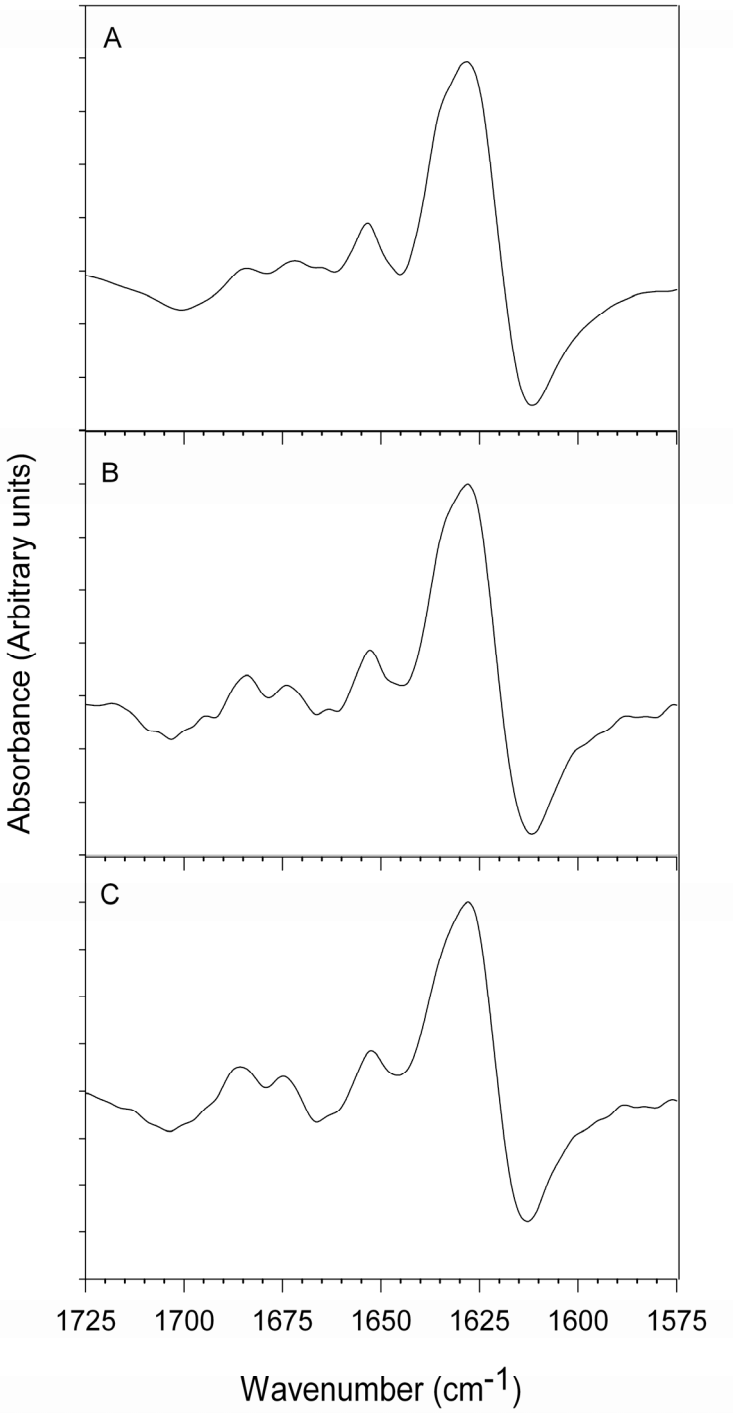


Figure 2. Second derivative FTIR spectra of the CsA crystalline control (A) and a solution of 10% (w/v) of CsA in 32% MeOH/32% ACN/36% (w/v) H_2O (B). Peak wavenumbers in the crystalline control (A): 1624 (antiparallel β-sheet), 1636 (loose β-sheet), 1653 (γ-loop), 1674 (type-II β-turn), 1688 (MeBMT-turn) and 1695 cm^{-1} (aggregate β-sheet); peak wavenumbers in the co-solvent solution (B): 1630 (β-sheet), 1663 (γ-turn) and 1678 cm^{-1} (type-II β-turn). See also Table 2.

If the type of solid dispersion of CsA and inulin would be a solid solution, then the amide groups of CsA would be intermolecularly oriented. Therefore in a solid solution, the FTIR spectrum of CsA should have the β-sheet band at high wavenumbers (~1630 cm^{-1}), contain a γ-turn band (~1663 cm^{-1}), and the MeBMT-turn band (~1685 cm^{-1}) should be absent. In contrast, if the type of solid dispersion would be a solid suspension, then the amide groups would be intramolecularly oriented, resulting in an FTIR spectrum with a β-sheet band at low wavenumbers (~1624 cm^{-1}), a γ-loop band (~1653 cm^{-1}) and the MeBMT-turn band at ~1685 cm^{-1}. A combination of a solid solution and a solid suspension should contain variable amounts of each structural feature, depending on the drug load.
CsA in the inulin solid dispersions (Figures 3A to 3D) apparently has few outward oriented amide groups as all solid dispersions, as well as pure spray-freeze dried CsA (Figure 3E), contain a β-sheet, γ-loop, and MeBMT-turn. The peak position of the β-sheet band of the solid dispersions and pure spray-freeze dried CsA is in between of the peak positions seen in the crystalline state and in the co-solvent solution (Table 2). Furthermore, the presence of the γ-loop and the absence of the γ-turn in the secondary structure indicates that CsA adopts a cis orientation between $MeLeu^{9}$ and $MeLeu^{10}$. Finally, the presence of the MeBMT-turn indicates that the $MeBMT^{1}$ side chain is in the proximity of the backbone. At higher drug loads, the relative intensity of the MeBMT-turn seems to increase.

Based on the data obtained by measurement of the secondary structure of CsA in the solid dispersion, the type of solid dispersion can best be described as a combination of a solid solution and suspension. Firstly, the peak position of the β-sheet band of the solid dispersions and pure spray-freeze dried CsA indicates that the type of solid dispersion is a combination of a solid solution and suspension, i.e. CsA is present in a molecular distribution and as clusters. Secondly, the presence of the γ-loop and corresponding cis orientation between $MeLeu^{9}$ and $MeLeu^{10}$ suggests an intramolecular orientation of $D\text{-}Ala^{8}NH$, which suggests a lipophilic environment. Thirdly, proximity of the $MeBMT^{1}$ side chain to the backbone suggests the presence of CsA clusters. Finally, the increasing intensity of the MeBMT-turn suggests that powders with higher drug loads contain more CsA clusters. Therefore, the amount of CsA in the form of solid suspension increases at higher drug loads.


A
B
C
Absorbance (Arbitrary units)
1725
1700
1675
1650
1625
1600
1575
Wavenumber (cm-1)

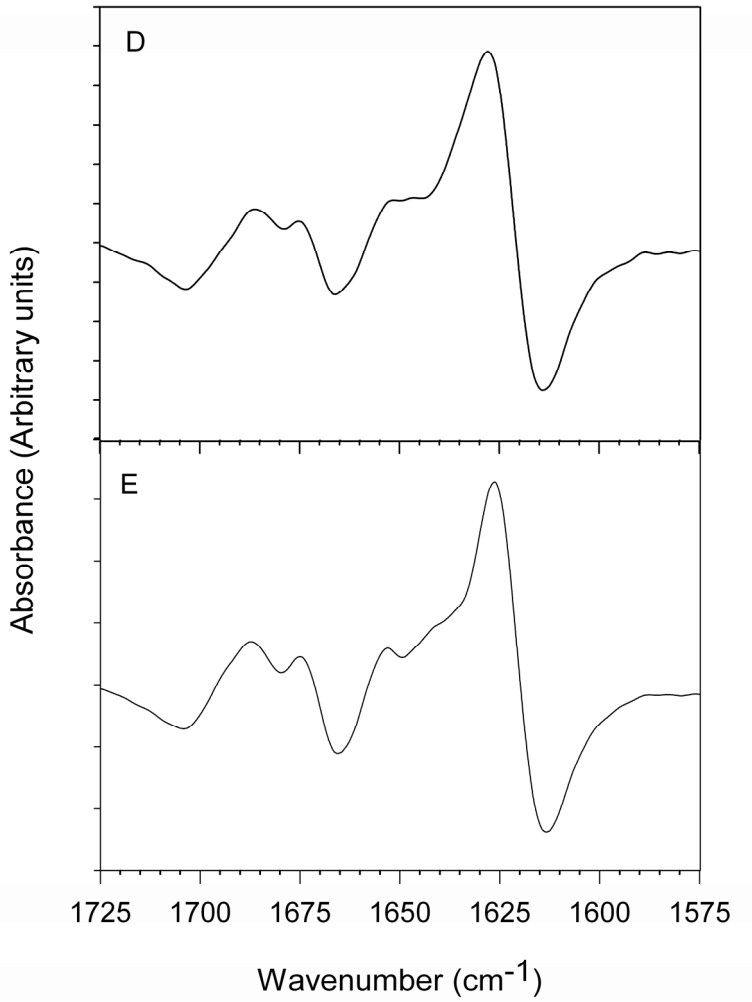


Figure 3. Second derivative FTIR spectra of the 10% (A), 20% (B), 30% (C), 50% (D) w/w solid dispersions and pure spray freeze dried CsA (E). Peak wavenumbers: 1626 - 1628 (β-sheet), 1653 (γ-loop), 1672 - 1676 (type-II β-turn) and 1684 - 1688 cm^{-1} (MeBMT-turn). See also Table 2.

Table 2. Peak positions of structural elements in the secondary structure of Cyclosporine A.

Data from Stevenson et al. (34)							
Sample	ß-sheets [*1]		γ-loop	γ-turn	Type-II ß -turn	MeBMT turn	Aggregate ß-sheet
Co-solvent solution	-	-	-	1661	1679	-	-
Spray-dried from ethanol	1624	1638	1653	-	1673	1686	-
Crystalline	1624	-	1645	-	1672	1683	-

Data from this study							
Sample	ß-sheets [*1]		γ-loop	γ-turn	Type-II ß -turn	MeBMT turn	Aggregate ß -sheet
Co-solvent solution	1630	-	-	1663	1678	-	-
10% w/w	1628	-	1653	-	1672	1684	-
20% w/w	1628	-	1653	-	1674	1684	-
30% w/w	1628	-	1653	-	1674	1684	-
50% w/w	1628	-	1653	-	1676	1686	-
Pure spray-freeze dried CsA	1626	1636	1653	-	1674	1688	-
Crystalline	1624	1636	1653	-	1674	1688	1695

[*1] In CsA two β-sheets occur: an antiparallel β-sheet (1624 cm^{-1}) and a looser β-sheet (1636 cm^{-1}) (37). In some samples both β-sheets overlap, resulting in a single β-sheet band at a wavenumber between 1624 and 1636 cm^{-1}.

The percentage of solid solution and solid suspension can be quantified by examination of the unresolved amide I bands. The amide I band depends on the orientation of the amide groups as shown in the secondary structure. The amide I band in the crystalline state clearly differs from the amide I in the co-solvent solution (Figure 4A). Furthermore, the amide I band of CsA in the inulin solid dispersion depends on the drug load (Figure 4B). As can be seen, the amide I band becomes more similar to the amide I band of the crystalline state with increasing drug loads.

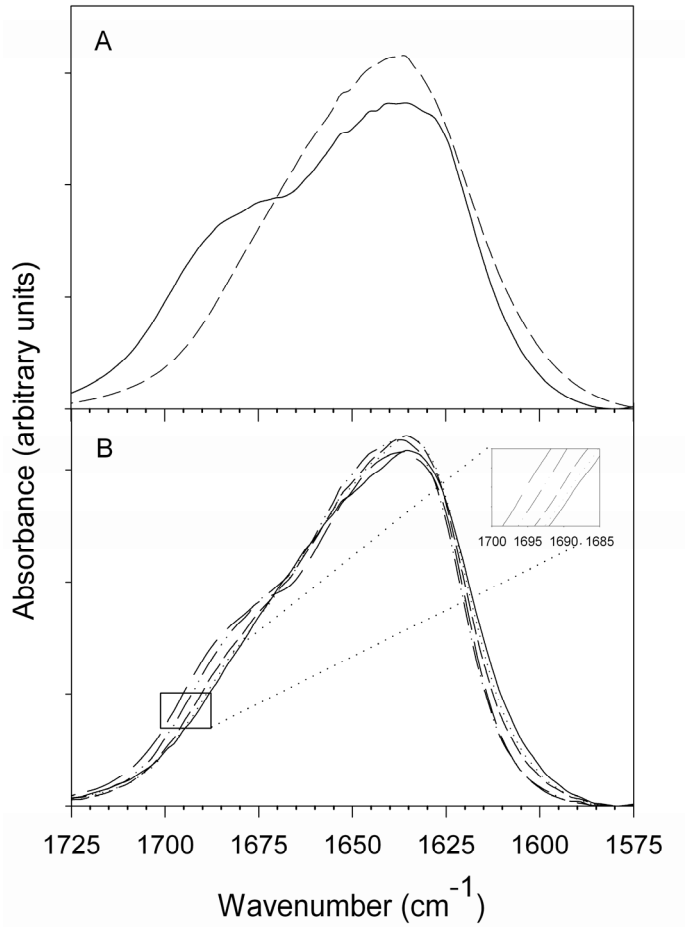


Figure 4. Base-line corrected and normalized amide I peak from FTIR spectra of CsA. (A) controls of crystalline CsA (——) and in MeOH/ACN/H_2O (— —). (B) solid dispersions containing 10% w/w (——), 20% w/w (·····), 30% w/w (— —) and 50% w/w (— ·· — ·· —) CsA, and pure spray freeze dried CsA (—— ——).

By calculating the percentage of area-overlap of the different amide I bands relative to the amide I band of CsA in the co-solvent solution, it is possible to quantify the differences caused by an inter- to intramolecular change in rotation of the amide groups (Figure 5). CsA dissolved in the co-solvent solution mainly has an intermolecular orientation of the amide groups as shown by analysis of the secondary derivative. In contrast, CsA

in the crystalline state mainly has intramolecular orientation of the amide groups. Similar to the secondary structure, the percentage area overlap of the amide I band of both CsA in the solid dispersion and pure spray freeze dried is in between those of CsA in the co-solvent solution and crystalline CsA.

The 10% w/w solid dispersion already has 3.8% difference in percentage area overlap in comparison with CsA in the co-solvent solution, indicating that at a drug load of 10% relatively much of the amide groups are oriented intramolecularly. The difference in percentage area overlap increases up to the 50% w/w solid dispersion. The 50% w/w solid dispersion has a difference in percentage area overlap which is nearly the same as for pure spray-freeze dried CsA, indicating that the majority of the amide groups are intramolecularly orientated. Pure spray freeze dried CsA has a difference in percentage area overlap of 8% with CsA in the co-solvent solution.

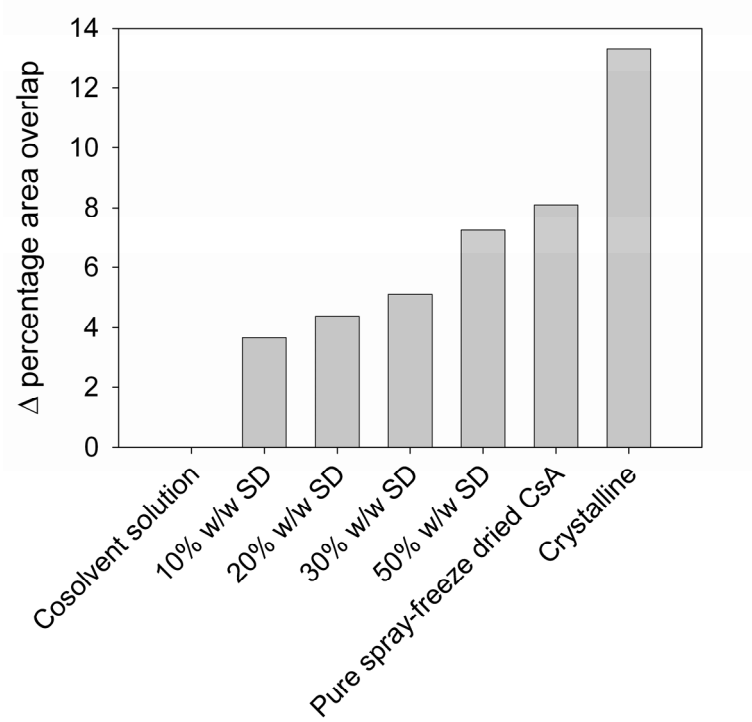


Figure 5. Difference in percentage area overlap of the amide I bands compared to the amide I band of CsA in the co-solvent solution.

If on the one hand pure spray freeze dried CsA is assumed to represent a complete solid suspension and on the other hand CsA dissolved in the co-solvent solution to represent a complete solid solution, then it is possible the calculate the percentage of each type of solid dispersion. However, the polarity of the co-solvent solution and amorphous inulin presumably differs. As a result, the ability to establish orientation of the amide groups of CsA by the co-solvent solution in comparison with inulin may therefore be different. The calculation thus only gives an indication about the percentage of type of solid dispersion. The 10% w/w solid dispersion has a 3.7% difference in percentage area overlap. When compared to the 8.1% difference in area overlap of pure spray freeze dried CsA, which was assumed to represent a complete solid suspension, it follows that the 10% w/w solid dispersion contains 55% of CsA as a solid solution and 45% of a solid suspension. The 20%, 30% and 50% w/w solid dispersion would then contain 54%, 63% and 90% of the CsA as a solid suspension, respectively.

Aerosol characteristics

Spray freeze drying results in log-normal geometrical particle size distributions with relatively large volume median diameters (x_{50}) (Table 3). The GSD of the spray freeze dried solid dispersions, as calculated with equation 1, are between 1.87 and 2.97 (Table 3).

In order to evaluate the aerodynamic behavior and measure the mass median aerodynamic diameter (MMAD), the powder was analyzed with cascade impactor analysis. As the powder showed rather cohesive behavior, a test inhaler which is able to generate relatively high deagglomeration forces was used (31).

Table 3. Particle size analysis: x_{16}, x_{50} and x_{84} undersize values of the cumulative geometric size distributions, MMAD and GSD of the spray freeze dried powders.

Formulation	x_{16} (µm)	x_{50} (µm)	x_{84} (µm)	GSD (-)	MMAD (µm)
10% w/w CsA	3.08	7.10	17.10	2.36	-
20% w/w CsA	3.53	7.29	15.29	2.08	-
30% w/w CsA	3.52	6.84	12.32	1.87	3.50
50% w/w CsA	2.46	8.44	21.74	2.97	3.39
Pure spray-freeze dried CsA	2.94	8.81	19.43	2.57	-

Both the 30 and 50% solid dispersions exhibit excellent inhalation characteristics (Figure 6). The spray freeze dried CsA solid dispersions show a low inhaler retention of ~ 4%, which leads to a high emitted fraction of ~ 96% for both solid dispersions (Figure 6). The solid dispersion powders furthermore show good dispersion characteristics as indicated by the respirable fraction of higher than 75%, as calculated by the sum of deposition on stages 2, 3 and 4 plus the filter. Furthermore, the FPF is higher than 50% of the metered dose, indicating that the solid dispersions are suitable for inhalation.

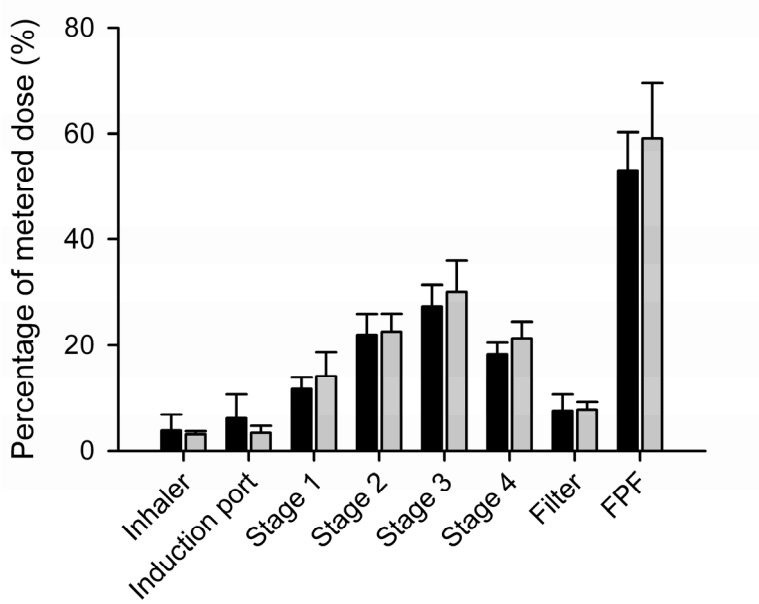


Figure 6. Cascade impactor analysis of the 30% (black bars) and 50% (grey bars) w/w solid dispersion at a flow rate of 60 l/min through an air classifier-based test inhaler. Error bars represent standard deviations (n=3).

Since a volume median diameter (x_{50}) is measured with laser diffraction and the particles were found to be spherical (scanning electron microscopy, data not shown), we can assume that d_e equals x_{50} as obtained with laser diffraction. The MMAD can be measured by using the mean of the cut off diameters (i.e. 4.95 and 2.4 µm for 3rd and 4th stage, respectively, as calculated according to the European Pharmacopeia (39)) and cumulative mass percentage (40). The intercept at the 50% mass

percentile of the cumulative mass percentage with cut off diameter corresponds to the MMAD. Thus, we can rewrite eq. 2 to:

$$MMAD = x_{50}\sqrt{\rho_p} \qquad (3)$$

In other words, by comparing the data obtained from laser diffraction and cascade impactor analysis, we can calculate the true particle density. The true particle density can be determined from the MMAD and volume median diameter if a log-normal distribution, a dynamic shape factor of 1 and a particle size independent density is assumed (equation 3). The measured MMADs are 3.50 and 3.39 µm for the 30% and 50% drug load solid dispersions, respectively (Table 3). Given equation 3 it is possible to combine laser diffraction ($x_{50}=d_e$) with cascade impaction data (MMAD) to calculate the density (ρ_p) of the particles. The comparison results in a calculated density of 0.26 and 0.16 g/cm^3 for the 30% and 50% drug load solid dispersions, respectively. Since the true densities of CsA and inulin are around 1.5 g/cm^3, the calculated density indicates the high porosity of the spray freeze dried particles.
Spray freeze drying of CsA and inulin resulted in relatively large (x_{50} ~ 7.5 µm) but porous (low density) particles. In literature (40-43), several advantages are ascribed to the use of (large) porous particles for pulmonary administration such as high emitted dose (fraction that is released from the inhaler), good dispersion characteristics and high – deep – lung deposition, which was confirmed in this study.

Dissolution
The dissolution rate of CsA formulated as a solid dispersion was significantly increased compared to the physical mixture, which consisted of pure spray freeze dried CsA (Figure 7) and spray freeze dried inulin. In the 10% and 20% w/w CsA-containing solid dispersions, inulin dissolves slightly faster than CsA (Figure 7A). The faster dissolution of inulin indicates that inulin dissolves first, followed by CsA. All solid dispersions dissolve faster than the physical mixture, albeit without a clear correlation between drug load and dissolution rate (Figure 7B). After 30 minutes all solid dispersions have dissolved for more than 80% compared to only 50% of the physical mixture.

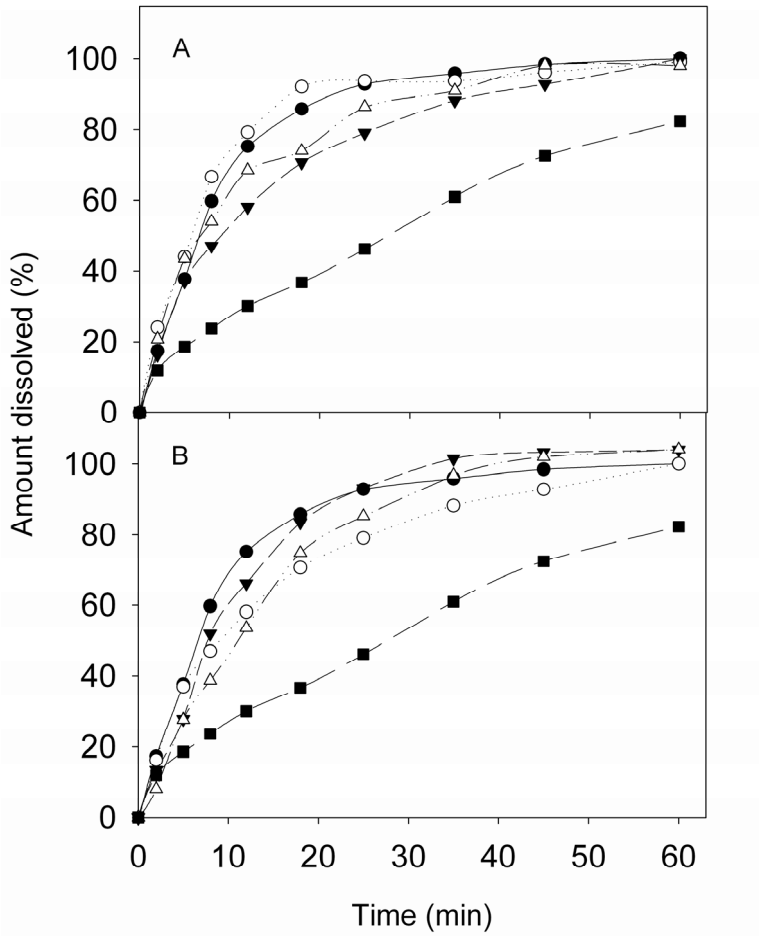


Figure 7. Dissolution behavior of the solid dispersions. (A) Dissolution behavior of a 10% (represented by circles) and 20% w/w (represented by triangles) solid dispersion. Open symbols represent inulin, closed symbols represent CsA. Squares represent the dissolution behavior of CsA from the physical mixture (30 mg CsA + 70 mg inulin). (B) Dissolution of CsA from the 10% (black circles), 20% (white circles), 30% (black triangles), 50% (open triangles) solid dispersion and from the physical mixture (squares).

The improved dissolution rate of the solid dispersion powder can be ascribed to the presence of inulin in the solid dispersions for two reasons. Firstly, the higher specific surface area of the solid dispersions, compared with pure spray freeze dried CsA, will have contributed to the higher

dissolution rate. Secondly, inulin improves wetting of CsA, even at drug loads up to 50%.

CONCLUSION

In this study a dry powder formulation containing CsA was developed for local administration in lung transplant patients. The presented dry powder formulations contain large porous particles which dissolve rapidly, even at high drug loads. The type of dry powder formulation was a solid dispersion. The solid dispersion, as investigated with FTIR, was found to be a mixture of a solid solution and a solid suspension, the ratio of which depended on the drug load. For inhalation the high drug load formulations are especially favorable, since with these a lower amount of powder has to be inhaled by the patient. Future studies will be performed to investigate whether this powder is a feasible alternative to nebulization and is able to improve therapeutic efficacy of treatment of lung transplant patients without increasing local and systemic side effects.

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CHAPTER 4A

The efficacy of a new pulmonary cyclosporine A powder formulation in the prevention of transplant rejection in rats

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ABSTRACT

***Background*. The aim of this pilot study was to determine the pharmacokinetics of cyclosporine A powder for inhalation (iCsA) and its efficacy in preventing rejection in experimental lung transplantation in rats.**

***Methods*. Single dose pharmacokinetics (10 mg/kg) of pulmonary and orally administered cyclosporine A was determined in whole blood and in lung and kidney tissue. The efficacy of iCsA (2.5 and 5 mg/kg) in inhibiting rejection was determined in an orthotopic left lung transplantation rat model and compared with orally administered CsA (5 and 10 mg/kg). The ventilation score of lung allografts was assessed with roentgenograms. At day 10 post-operative, the rats were terminated and lungs were prepared for histological analysis.**

***Results*. In the pharmacokinetic study, AUC_{0-48} values in blood for iCsA and oral CsA were similar (47,790±1,739 and 46,987±2,439 $ng \cdot h \cdot mL^{-1}$, respectively). In contrast, iCsA levels in lung tissue were much higher than oral CsA levels (AUC: 9,152,977±698,920 versus 84,149±8,134 $ng \cdot h \cdot g^{-1}$, respectively), showing the effectiveness of the pulmonary administration. In the rejection study, non-treated animals showed complete rejection after 8 days on roentgenograms. Treatment with 5 mg/kg iCsA reduced rejection on day 10, while the 2.5 mg/kg dose did not inhibit rejection. Oral CsA 10 mg/kg strongly reduced rejection, while the 5 mg/kg dose showed hardly any effect on rejection.**

***Conclusions*. We conclude that iCsA is an effective immunosuppressive formulation, which might become a valuable asset for clinical use in combination with systemic immunosuppression.**

INTRODUCTION

Lung transplantation is an effective treatment for end-stage lung disease, since it improves quality of life and prolongs survival. Oral treatment with calcineurin inhibitors such as cyclosporine A (CsA) is a cornerstone of the current immunosuppressive strategies. To reduce the notorious adverse effect profile of CsA, newer strategies are employed such as exchanging CsA for Tacrolimus (1), improved pharmacokinetic monitoring and subsequent dosing based on the 2 hours post-dose CsA concentration (C2) instead of the trough (C0) concentration (2-5), delayed initiation of CsA therapy through induction therapy with Basiliximab (6), combination therapy with Everolimus (Certican®) to allow a lower CsA dose with similar efficacy and less adverse effects (7-12) or pulmonary delivery of CsA (13, 14). Pulmonary delivery of CsA by nebulization has shown to improve the therapeutic efficacy in lung transplantation in animal models (15-17) and humans (14, 18-20). In humans, aerosolized CsA resulted in lower rejection rates in patients with acute (13) and chronic (14, 20) rejection, resulting in improved survival.

In the clinical studies, a propylene glycol solution that contained CsA was used (13, 18, 20, 21). Unfortunately, inhalation of the CsA solution was accompanied by severe complications. First, patients had to be pretreated with aerosolized lidocaine and albuterol to reduce side effects like dyspnea, cough and pharyngeal soreness (14). Secondly, nebulization resulted in a lung deposition of only 5.4 - 11.2% of the metered dose in the lung (21). Since Corcoran showed that doses of at least 5 mg CsA are needed in the peripheral part of the lung to obtain a significant improvement in lung function (18), it is clear that with conventional administration, high nebulized doses are necessary for effective treatment. As detailed above, higher dosing is limited by local side effects.

To avoid the difficulties faced with propylene glycol nebulization, we developed CsA as a powder for inhalation (iCsA) using an inulin-based solid dispersion formulation technique (22). A major advantage of dry powder inhalation over fluid nebulization is that no solvent is needed, which could make the premedication redundant. Other advantages are the higher pulmonary deposition efficiency and ease of administration (23).

Since this is the first study of iCsA in animals, the purpose of this study was to investigate 1) single dose pharmacokinetics for dose determination

in the efficacy part of this study and 2) the effectiveness of iCsA in preventing rejection in orthotopic left-lung transplantation in rats.

MATERIALS AND METHODS

Materials

Neoral® was obtained from Novartis, Basel, Switzerland. Cyclosporine as a powder for inhalation (iCsA) was compounded at the Hospital Pharmacy of the University Medical Center Groningen (UMCG) as described previously (22).

Animal experiments

Brown-Norway (BN) and Albino-Oxford (AO) male rats (200-260 gram) were purchased from Harlan (Horst, the Netherlands). Our studies conformed to the Dutch Law on Experimental Animal Care and were approved by the Institutional Animal Care and Use Committee of the University of Groningen. During all experimental procedures, rats were under isoflurane anesthesia.

Single dose (10 mg/kg) pharmacokinetics (iCsA) and oral CsA (Neoral®, Novartis, Basel, Switzerland) were determined in BN rats (n=18 per treatment). iCsA was administered by insufflation (model DP-4, Penn-Century, Philadelphia, PA, USA) after orotracheal intubation. Oral CsA was administered undiluted by oral gavage. Blood was collected at t= 0, 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 hours after administration; kidneys and lungs were harvested at t= 0, 2, 6, 12, 24 and 48 hours.

Orthotopic, left lung transplantation was performed across a major histocompatibility barrier with AO ($RT1^u$) as donor and BN ($RT1^n$) as recipient (24, 25). In total, twenty-one transplantations were performed (table 1). Rats were treated with 2.5 or 5 mg/kg iCsA on day 0, 2, 4, 6 and 8. Reference treatment was oral CsA in a dose of 5 or 10 mg/kg. Isogenic and allogenic transplantation served as untreated transplantation controls. Non-transplanted BN rats served as treatment controls and received 5 mg/kg iCsA or 10 mg/kg oral CsA. Determination of systemic CsA trough levels and ventilation score was performed at day 2, 4, 6, 8 and 10. At day 10, lungs were collected for histological analyses.

Table 1. Experimental groups in lung allograft rejection study.

Group	n	Transplantation (Donor-recipient)	Treatment	Dose (mg/kg)	Ischemia time (min ± *SD*)
1	5*	AO-BN	iCsA	2.5	34.2 ± 5.2
2	4*	AO-BN	iCsA	5	32.7 ± 1.5
3	3	AO-BN	Oral CsA	5	33.0 ± 3.5
4	3	AO-BN	Oral CsA	10	34.0 ± 1.7
5	3	BN-BN	-	-	37.0 ± 7.9
6	3	AO-BN	-	-	37.3 ± 4.0
7	3	BN	iCsA	5	-
8	3	BN	Neoral, orally	10	-

* In group 1, 2 animals died during iCsA treatment and 1 animal died during the transplantation procedure. In group 2, 1 animal died during iCsA treatment. Autopsy revealed that the three deaths during treatment of iCsA were caused by destruction of the larynx as a consequence of the intubation procedure.

Analyses

Cyclosporine concentrations were determined using a fluorescence polarization immunoassay (AxSym®, Abbott Laboratories, Chicago, USA) (26). CsA blood levels were fitted with first-order kinetics (equation 1), followed by calculation of pharmacokinetic parameters:

$$C(t) = \left(\frac{C_0 k_a}{k_a - k_{el}} \right) \cdot e^{-k_{el} \cdot t} - \left(\frac{C_0 k_a}{k_a - k_{el}} \right) \cdot e^{-k_a \cdot t} \qquad (1)$$

where C(t) is the concentration in the compartment (ng/mL), C_0 a constant (ng/mL) representing the maximum compartment concentration obtained by only absorption at t=0 and k_a and k_{el} the absorption and elimination rate constants, respectively (h^{-1}). Bailer's method was used to calculate the area under the curve (AUC) and standard error from 0 to 48 hours (27).

CsA tissue levels were determined by mixing an aliquot EDTA blood with a known amount of tissue sample extract and processed similarly to a whole blood sample.

Rejection was determined by two blinded and independent observers on basis of roentgenograms and histology (24, 25). Ventilation scores of roentgenograms ranged from 6 (normal looking lungs) to 0 (opaque, fully

rejected lungs). Histological scores ranged from 0 (normal looking lungs) to 4 (completely rejected lungs).

Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM), unless mentioned otherwise. Statistical comparisons between groups were performed by the Wilcoxon-test. Statistical significance was taken as $p<0.05$.

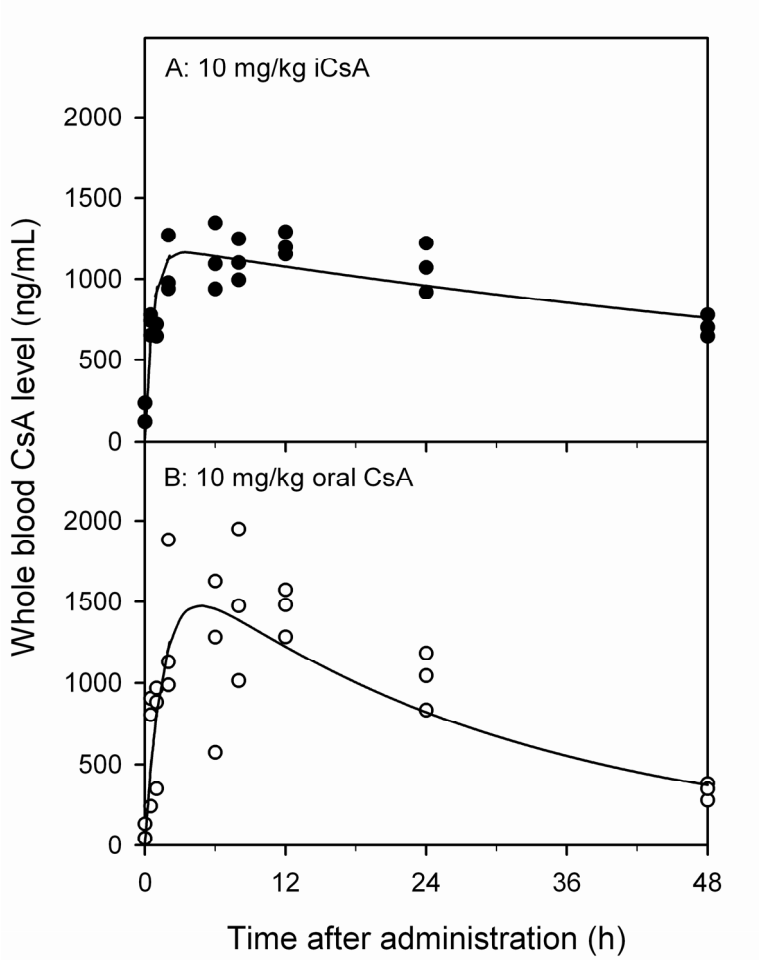

Figure 1. Cyclosporine whole blood levels after single 10 mg/kg dose. (A) iCsA 10 mg/kg and (B) oral CsA 10 mg/kg. Each circle represents an individual data point and lines represent the fit of the data points.

RESULTS

Pharmacokinetic study

Inhalation and oral administration of CsA resulted in substantial systemic blood levels (figure 1). Insufflation of CsA (figure 1A) led to a lower variation of individual whole blood levels compared to orally administered CsA (figure 1B).

Table 2. Summary of fitting parameters, calculated C_{max}, t_{max} and $t_{½el}$ by using eq. 1 and Calculated area under the curve in whole blood, kidney and lung tissue. Targeting index was calculated by dividing tissue AUC's by whole blood AUC. C_0 is a constant (ng/mL) representing the maximum compartment concentration obtained by only absorption at t=0, k_a the absorption rate constant and k_{el} the elimination rate constant.

Administration	iCsA Insufflation	Neoral Oral gavage
Dose (mg/kg)	10	10
Results of fit		
C_1 ($ng \cdot ml^{-1}$)	1,201.59	1,727.05
k_a (h^{-1})	1.474	0.653
L_1 (h^{-1})	0.00968	0.03309
Pharmacokinetic parameters		
C_{max} ($ng \cdot ml^{-1}$)	1,162.32	1,472.78
t_{max} (h)	3.4	4.8
$t_{½el}$ (h)	71.6	20.9
AUC_{0-48}		
Whole blood ($ng \cdot h \cdot ml^{-1}$ ± *SD*)	47,790 ± *1,739*	46,987 ± *2,439*
Kidney ($ng \cdot h \cdot g^{-1}$ ± *SD*) *	166,679 ± *8,371*	130,962 ± *8,541*
Lung ($ng \cdot h \cdot g^{-1}$ ± *SD*) *	9,152,977 ± *698,920*	84,149 ± *8,134*
Targeting index		
Kidney (-)	3.5	2.8
Lung (-)	191.5	1.8

* Significant difference, P<0.05

Pharmacokinetic parameters (table 2) were calculated using equation 1, and curve fitting resulted in the levels given in figure 1. Inhalation of iCsA resulted in similar whole blood AUC_{0-48} as oral CsA (table 2), despite a lower peak level (C_{max} of 1,162 vs. 1,472 ng/mL), which was reached

earlier (t_{max} of 3.4 vs. 4.8 hours) (table 2). Furthermore, the elimination half-time in blood ($t_{½el}$) of iCsA was substantially longer than of oral CsA (72 vs. 21 hours, respectively). The low t_{max} demonstrates that CsA after inhalation is rapidly absorbed into the systemic circulation, while the long $t_{½el}$ suggests that CsA is slowly released from the lungs.

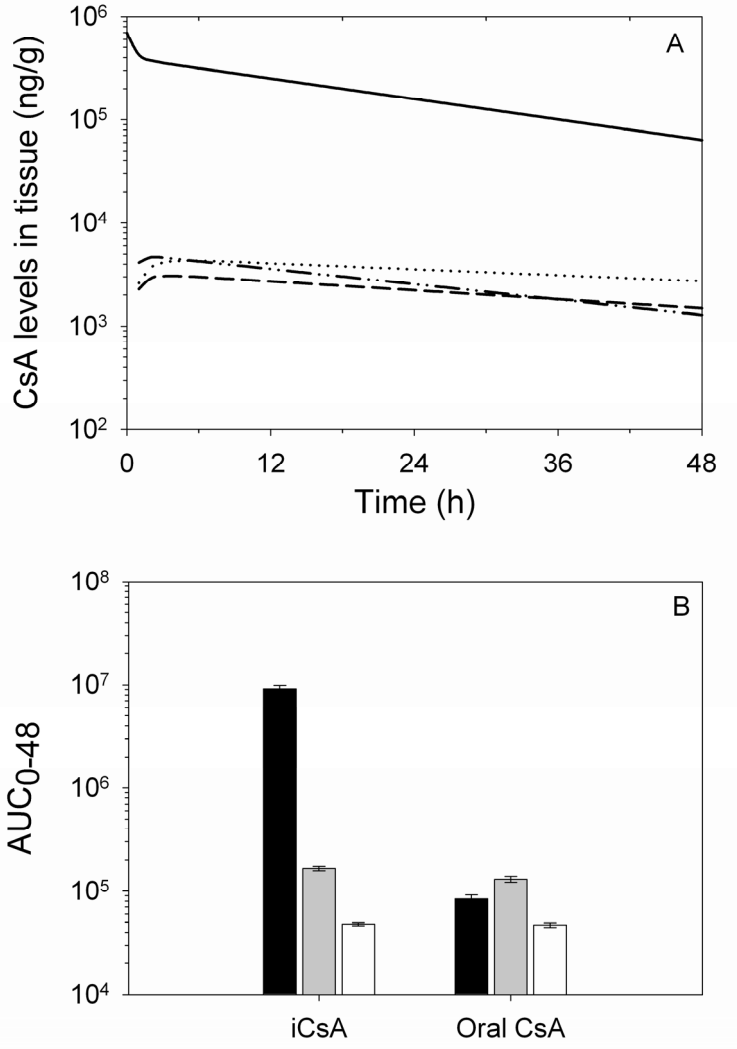

Figure 2. (A) CsA tissue levels after administration of 10 mg/kg iCsA (lung: ———; kidney: ••••••••) and oral CsA (lung: — — —; kidney: — •• —). (B) AUC0-48 of CsA in tissue (lung: black bars; kidney: grey bars) and blood (white bar). Error bars represent standard deviation calculated according to Bailer's method.

The small difference of the AUC_{0-48} in blood for iCsA and oral CsA suggests that the absolute bioavailability in the systemic compartment was similar over a 48-hour period.
In contrast, iCsA resulted in a 109-fold higher lung availability (AUC_{0-48}) compared to oral CsA (table 2 and figure 2). During the complete period, the lung tissue level after insufflation of iCsA was much higher than after oral CsA (figure 2A). In kidney tissue, the AUC_{0-48} was 27% higher for iCsA than for oral CsA, demonstrating that the lung:kidney ratio was much higher for iCsA than for oral CsA. The increased availability of iCsA in the lung after insufflation is clearly seen in figure 2B. The lung targeting index, calculated by dividing lung tissue AUC by whole blood AUC, was 191.5 and 1.8 for iCsA and oral CsA, respectively, again demonstrating that insufflation of iCsA has higher availability in the lung compared to oral CsA administration.

Allograft rejection study

Ventilation

Treatment of transplanted animals with iCsA as monotherapy (groups 1 and 2) prevented rejection up to 10 days, but only at a 5 mg/kg dose (figure 3A). Up to 6 days post transplant, no difference between the 2.5 and 5 mg/kg dose was observed while after 6 days a substantial decrease in ventilation score was observed in the 2.5 mg/kg group, leading to full loss of ventilation at day 10. Compared to allogenic transplantation without treatment (group 6), inhalation of 5 mg/kg iCsA was effective in maintaining ventilation ($p=0.026$), indicating that monotherapy with iCsA inhibits the underlying rejection. Inhalation of 2.5 mg/kg did not differ significantly from allogenic transplantation without treatment.
Treatment with 5 or 10 mg/kg oral CsA (groups 3 and 4) prevented rejection compared to allogenic transplantation ($p=0.01$ and $p=0.002$, respectively) (figure 3B). The higher dose (10 mg/kg) was more effective than 5 mg/kg in preventing rejection ($p=0.01$). Treatment with 5 or 10 mg/kg oral CsA did not differ from treatment with 5 mg/kg iCsA (group 2) ($p=0.352$ and $p=0.937$, respectively).
In isogenic control animals (group 5), orthotopic left lung transplantation resulted in a reversible ischemia-induced decrease in the ventilation score at post-operative day 2, which surprisingly was especially seen in the untreated isogenic control group (figure 3C).

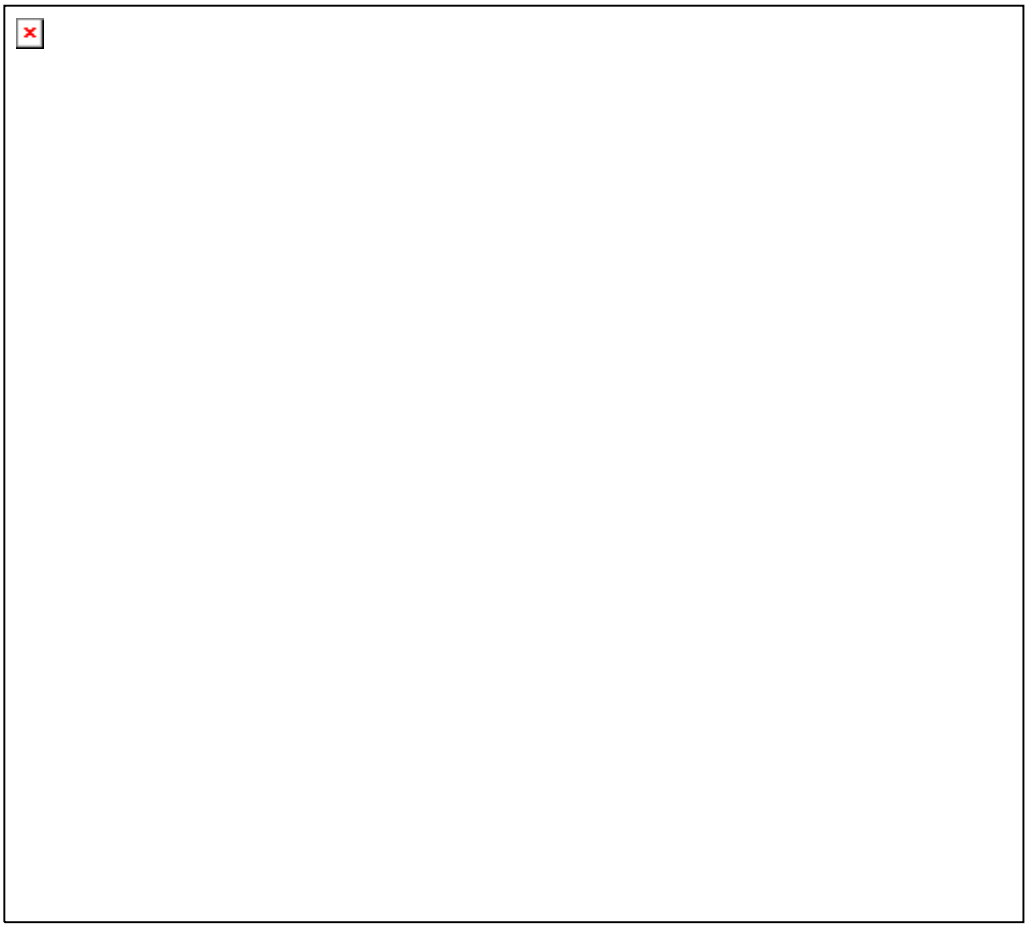

Figure 3. Rejection of the left lung as measured with ventilation scores (6: normal looking lungs; 0: opaque, fully rejected lungs) and histologic rejection score (0: normal looking lungs; 4: fully rejected lungs). (A) Ventilation score of animals that received allogenic transplantation and were treated with iCsA (black squares: 5 mg/kg; open squares: 2.5 mg/kg). (B) Ventilation score of animals that received allogenic transplantation and were treated with oral CsA (black triangles: 10 mg/kg; open triangles: 5 mg/kg). (C) Control animals that did not receive cyclosporine (black circles: isogenic transplantation BN-BN; open circles: allogenic transplantation AO-BN). (D) Histologic rejection score at day 10 post-operative. Error bars represent SEM (n=3). Statistically significant difference: allogenic control versus 5 mg/kg iCsA (*; P=0.026), allogenic versus 5 mg/kg (***; P=0.01) and 10 mg/kg oral CsA (**; P=0.002), isogenic versus 2.5 mg/kg iCsA (†; P=0.01) and isogenic versus 5 mg/kg oral CsA (††; P=0.01).

After 10 days, the ventilation score normalized. In allogenic left lung transplantation without treatment (group 6), the ventilation score decreased to 0 at day 8 after transplantation, which indicated complete rejection after 8 days. Despite full loss of ventilation of the left lung, the rat functioned normally due to the unaffected right lung. The non-transplanted groups treated with 5 mg/kg iCsA (group 7) or 10 mg/kg oral CsA (group 8) did not show any loss of ventilation (data not shown), proving that the treatment itself did not have an influence on the ventilation score.

The ventilation score of isogenic transplants didn't differ significantly from allogenic transplants treated with 5 mg/kg iCsA or 10 mg/kg oral CsA ($p=0.589$ and $p=0.699$, respectively). In contrast, isogenic transplantation was significantly different compared to 2.5 mg/kg iCsA ($p=0.01$) and 5 mg/kg oral CsA ($p=0.01$). These results show that monotherapy with 5 mg/kg iCsA or 10 mg/kg oral CsA effectively prevents rejection as measured by the ventilation score.

Histology

After 10 days the animals were sacrificed and the histology of the lungs was analyzed (figure 4). After treatment with 5 mg/kg iCsA, the allografts exhibited some perivascular, peribronchial and interstitial infiltrates, while the pulmonary architecture and alveolar integrity were preserved. On average, administration of 5 mg/kg iCsA resulted in a grade 2.7 rejection (figure 3D). The lower dose of 2.5 mg/kg iCsA was not effective in suppression of rejection (grade 4, figure 3D).

Oral administration of CsA in a 5 mg/kg dose resulted in almost complete rejection similar to 2.5 mg/kg iCsA but with edematous alveoli and more infiltrates. These allografts showed grade 3.5 rejection on average (figure 3D). At a 10 mg/kg oral dose, the allografts exhibited some perivascular inflammation and had a mild grade 1.3 rejection score.

Control recipients with isogenically transplanted lungs (group 5) showed no signs of rejection. In contrast, allografts from non-treated control recipients exhibited full rejection grade 4 rejection. The alveoli of the allografts were obliterated and contained necrotic debris. In addition, interstitial destruction and vascular thrombosis was observed. Histological analysis revealed that inhalation of 5 mg/kg iCsA in non-transplanted animals (group 7) resulted in some alveolar, perivascular and interstitial

reaction which differed from reaction typically seen with rejection. Focal infiltrates of macrophages, lymphocytes and polymorphous nuclear cells were visible (data not shown). However, this reaction was not visible on roentgenograms. The control animals that received 10 mg/kg oral CsA (group 8) did not show such signs of reaction in the lungs.

Systemic trough CsA blood levels

Trough CsA blood levels were determined in rats that received lung transplantation and were treated with iCsA or oral CsA systemic (figure 4). Generally, rats treated with 2.5 mg/kg iCsA had relatively lower trough levels (average 93±26 ng/mL) than 5 mg/kg iCsA (average 243±54 ng/mL). In line with the findings in the pharmacokinetic study, treatment with 5 mg/kg iCsA resulted in lower trough blood levels than treatment with 5 mg/kg oral CsA (average 439±207 ng/mL). Treatment with 10 mg/kg oral CsA led to highest systemic trough levels (average 687±258 ng/mL). Furthermore, treatment with iCsA led to more constant trough levels compared to oral treatment, a finding that was also observed in the pharmacokinetic study.

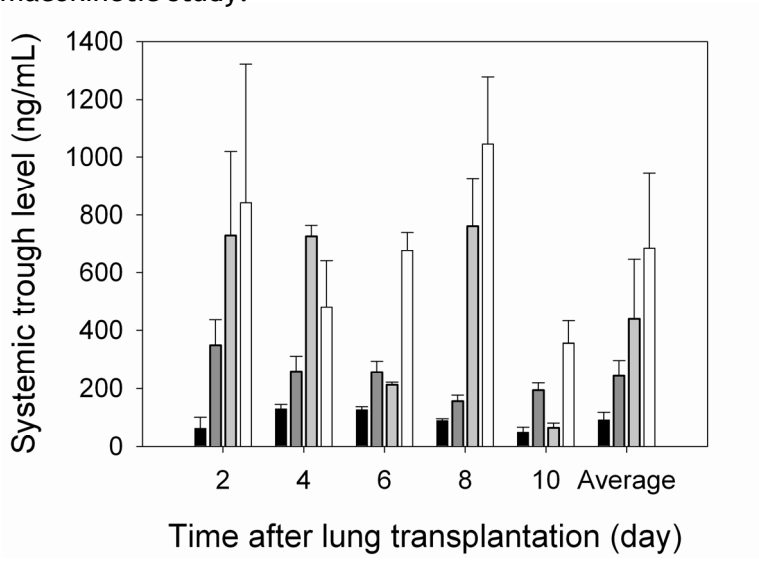

Figure 4. Systemic CsA trough levels of rats that received allogenic lung transplantation and were treated with 2.5 mg/kg iCsA (black bars), 5 mg/kg iCsA (dark grey bars), 5 mg/kg oral CsA (grey bars) or 10 mg/kg oral CsA (white bars). Time point "Average" represents the average systemic CsA trough level per group and calculated over all time points. Error bars represent SEM.

DISCUSSION

In this study, a new cyclosporine powder formulation suitable for pulmonary administration (iCsA) was effective in preventing rejection after lung transplantation in rats as single therapy. A trend was observed between suppression of rejection and CsA dose, irrespective of the administration route, with 10 mg/kg being most effective, followed by 5 mg/kg. A dose of 2.5 mg/kg was not effective in suppression of rejection. In equal doses (5 mg/kg), iCsA showed suppression of rejection at lower trough blood levels than orally administered CsA. Therefore, the efficacy of iCsA appears to be related to both systemic and pulmonary concentrations.

Inhalation of iCsA resulted in much higher lung tissue levels than oral CsA. The high lung tissue levels after insufflations may have led to local pulmonary reaction as observed in the 5 mg/kg iCsA control group. Such reaction was not reported previously and may be the consequence of the extremely high tissue levels. This reaction requires further investigation. In the literature (15-17, 28) lower doses for effective suppression of rejection have been found, albeit in different animal models. The 10 mg/kg oral CsA group (group 8) displayed normal lung histology without signs of pulmonary reaction. Therefore, less, or even no pulmonary reaction is expected when iCsA would be given in lower doses.

The difference in lung tissue levels after inhalation and oral administration (lung targeting index) were about 7- to 14-fold higher in this study than Blot et al. (15) and Mitruka et al. (29) previously reported, which may have been due to the different administration method used in this study. Both Blot et al. and Mitruka et al. used nose-only exposure to the aerosol, while in this study insufflation was used. With nose-only exposure, a small part of the dose is inhaled; of the inhaled dose a portion is possibly swallowed, leading to systemic absorption. In contrast, the complete dose is given to the lungs with insufflation, which would result in much higher deposition and lower systemic exposure. As a consequence, the different administration method may have contributed to the occurrence of the local reaction by enabling much higher local doses.

The pharmacokinetics show that iCsA was for the largest part absorbed in the lung. After t_{max} has been reached, the lung tissue level declines very slowly, as well as the corresponding blood levels as shown by the high $t_{½el}$. This indicates that CsA is only gradually released into systemic

compartment. The systemic blood levels after administration iCsA are more reproducible than after oral administration of CsA, which is the result of the less complicated route of entrance into the systemic compartment: directly from the lungs. In contrast, oral administration of CsA is influenced by gastrointestinal transit, food and first-pass metabolism, resulting in a reported bioavailability of 27% (30). Consequently, the availability in the target organ is much higher for iCsA.
The side effects of iCsA are limited. The kidney tissue level, was not different for iCsA and oral CsA and indicates comparable safety of both products. On the other hand, the ratio of lung to kidney tissue level (in fact an efficacy/safety parameter) is much higher for iCsA than for oral CsA. Therefore, iCsA as add-on therapy over conventional oral therapy would not drastically increase the systemic exposure and thereby the risk of nephrotoxicity.
To conclude, our study indicates that results of lung transplantation may be improved by combination therapy of iCsA with standard oral immunosuppressive therapy. In addition to other approaches to improve the efficacy/safety balance of immunosuppressive therapy with calcineurin inhibitors, iCsA may be an option. The different route of administration of iCsA in lung transplantation in combination with oral therapy may lead to more effective local immune suppression than can be reached by oral therapy only. Lower doses of iCsA would only have a limited effect on the systemic CsA levels, which would not increase the risk of side effects. Future studies should focus on establishing the safety of iCsA in pre-clinical studies and establishing the appropriate dose in combination with oral treatment.

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CHAPTER 4B

Cyclosporine A Solid Dispersion for Inhalation:

Effect of Dose on Pulmonary Reaction in Rats

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ABSTRACT

Background: **The aim of this study was to compare the pulmonary side-effects of high dose cyclosporine A solid dispersion for inhalation (iCsA) with low dose and with controls.**

Methods: **Rats were orotracheally intubated and given either 5 or 0.8 mg/kg iCsA by insufflation, 5 or 0.8 mg/kg placebo (inulin) powder by insufflation, insufflation without powder, or no insufflation. Dosing was performed on alternate days. On day 10, lungs were harvested for histological analysis.**

Results: **Insufflation of 5 mg/kg iCsA resulted in some alveolar, perivascular and interstitial reaction. The reaction after low-dose iCsA (0.8 mg/kg) did not differ from both placebo doses dose. Furthermore, the reaction observed after 0.8 mg/kg iCsA did not differ materially from untreated, but insufflated control groups.**

Conclusion: **The absence of an additional reaction after treatment with low-dose iCsA compared with placebo indicates that inhalation of iCsA is safe at dose levels of 0.8 mg/kg and lower.**

INTRODUCTION

Lung transplantation has become a therapeutic option for end-stage pulmonary disease. Oral treatment with calcineurin inhibitors, such as cyclosporine A or tacrolimus, is the cornerstone of current immunosuppression. However, calcineurin inhibitors are known for their side effects, such as neuropathy and nephrotoxicity, which narrows the therapeutic window (1-4).

Currently, calcineurin inhibitors are only available as formulations for parenteral or oral administration. After oral administration, the systemic concentration determines the tissue concentration in the target organ. To improve therapeutic efficacy, it has been suggested to increase the drug tissue levels in the lung via pulmonary administration. Iacono *et. al.* (5) have demonstrated in a single center study that liquid nebulization of CsA resulted in improved survival after lung transplantation compared with placebo. Unfortunately, patients had to be pretreated with local anesthetiscs to make the inhalation maneuver tolerable.

To improve the administration of CsA by inhalation and to avoid the pretreatment with local anesthetics, a dry powder formulation containing CsA (iCsA) has been developed based on inulin solid dispersion technology (6). In a previous study, the pharmacokinetics and efficacy of iCsA have been compared with oral administration of CsA (Neoral®) (7). iCsA resulted in much higher tissue levels in the lung than oral CsA, whereas the systemic exposure was similar. Furthermore, the lung tissue levels of iCsA remained higher for a longer period than those found after oral CsA. Inhalation of 5 mg/kg iCsA resulted in improved suppression of rejection in a transplantation model in rats compared to oral administration of 5 mg/kg CsA: on day 10 after transplantation, the ventilation score, which ranged from 6 (normal lungs) to 0 (opaque, fully rejected lungs), was 4.1 versus 2.5 for 5 mg/kg iCsA and oral CsA, respectively. The histologic score, which ranged from 0 (normal looking lungs) to 4 (fully rejected lungs), was 2.7 versus 3.5 for 5 mg/kg iCsA and oral CsA, respectively. However, inhalation of 5 mg/kg iCsA in control animals resulted in some alveolar, perivascular and interstitial reaction which differed from the reaction typically seen with rejection. Focal infiltrates of macrophages, lymphocytes and polymorphous nuclear cells were visible. This reaction was not visible on roentgenograms. The conclusion of that study was to use iCsA in combination with systemic treatment, but with a lower iCsA dose (7).

Therefore, the purpose of the current study was to investigate the (side-)effects of a lower iCsA dose and the effects of placebo (inulin) on the histology of the lung. Rats were treated according to the same protocol as in the study mentioned above. End point of this study was histologic analysis.

MATERIALS AND METHODS

Materials

Cyclosporine as a powder for inhalation (iCsA) and inulin (placebo) were formulated as described before (6). For iCsA, a co-solvent solution of tert-butanol and water (40/60 v/v) containing CsA and inulin was spray freeze-dried to obtain a solid dispersion of 50% w/w CsA in inulin (iCsA). The placebo powder was prepared similarly, only the inulin concentration was increased to have the same amount solid concentration.

Animals

Specific pathogen free, male Brown-Norway rats (BN/RijHSD), weighing 200 to 260 gram, were purchased from Harlan (Horst, the Netherlands). The rats were conventionally housed at the animal facility of the University of Groningen. The experimental protocol was approved by the institutional animal care committee of the University of Groningen. Prior to any experiment, the rats were allowed to acclimatize for a period of minimally 7 days. Throughout the experiment the rats were allowed to eat and drink ad libitum. The animals received human care in compliance with the Dutch Law on experimental animal care.

Methods

Administration

Rats were anesthetized with 4% isoflurane at an O_2 flow of 0.6 L/min and given atropine (0.05 mg/kg SC). Next, they were orotracheally intubated for the insufflation procedure. The powder for inhalation (iCsA) or placebo was administered by insufflation (Model DP-4, Penn-Century, Philadelphia, PA, USA).

Experimental groups and treatment regimen

All rats in this study were exposed to the same experimental procedures. All rats were anesthetized, followed by intubation. These procedures were repeated on alternate days for a period of 10 days. Depending on

the experimental group, additional insufflation with or without powder could be performed as detailed in Table 1.
We randomly divided rats into 6 Groups. Rats in Group 1 and 2 were given 5 mg/kg iCsA or 5 mg/kg placebo (inulin), respectively. Groups 3 and 4 were given 0.8 mg/kg iCsA or 0.8 mg/kg placebo, respectively. Rats in Group 5 and 6 served as controls: rats in Group 5 received insufflation without powder, while rats in group 6 were only intubated.
On day 10, all rats were euthanized by puncture of the aorta while they were under isoflurane anesthesia. The lungs were fixated with formalin, dissected and stored for histologic analysis.

Table 1. Experimental groups

Group	n	Treatment
1	3	5 mg/kg iCsA
2	4	0.8 mg/kg iCsA
3	4	5 mg/kg placebo
4	4	0.8 mg/kg placebo
5	4	Insufflation
6	4	-

Histologic analysis
The formalin-fixated lungs were embedded in paraffin and cut into 3 µm sections followed by hematoxylin-eosin (HE) staining. Sections were analyzed as previously described (8).

RESULTS
Insufflation of 5 mg/kg iCsA on alternate days for a duration of 10 days did not result in macroscopic (i.e. on X-rays) reactions, but as in our former experiment (7), did result in a measurable microscopic inflammatory reaction (Figure 1). Microscopically, focal infiltrates of macrophages, lymphocytes and polymorphous nuclear cells were visible in all rats treated with 5 mg/kg iCsA (Table 2).
In contrast, insufflation of 0.8 mg/kg iCsA using the same dosing scheme, only resulted in a minor reaction as compared to 5 mg/kg iCsA (Table 2): in most cases, the alveoli were clean, in one rat some alveolar macrophages were seen. In two rats, polymorphous nuclear cells were visible in the interstitium. In a single rat, some infiltrates were observed perivascularly, consisting of macrophages and giant cells.

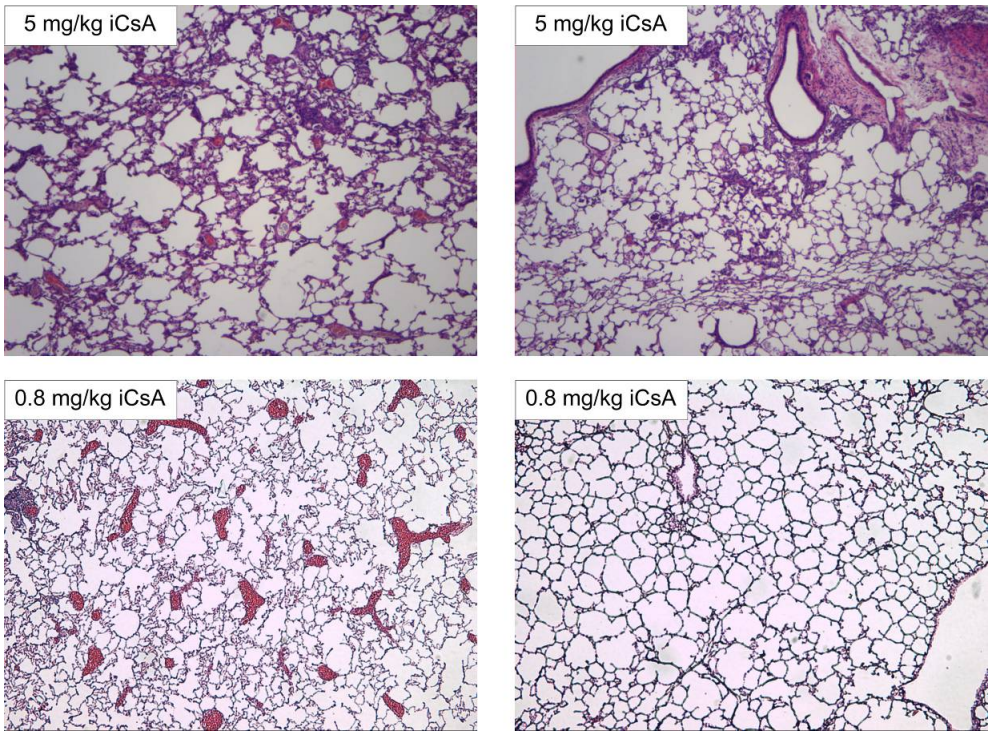

Figure 1. Hematoxillin and eosin stainings of lung tissue from rats treated with 5 mg/kg (upper part) and 0.8 mg/kg iCsA (lower part). Each representative photograph is taken from the lung of a different rat. Magnification was 4x for 5 mg/kg iCsA and 5x for 0.8 mg/kg iCsA doses.

In the control groups that received inulin (placebo) by insufflation of similar doses as iCsA (5 and 0.8 mg/kg), minor histologic reactions were also found. These were far less than observed after insufflation of 5 mg/kg iCsA insufflation (Figure 2), yet similar as 0.8 mg/kg iCsA insufflation (Table 2). At a 5 mg/kg inulin dose, a few small infiltrates consisting of polymorphous nuclear cells were perivascularly seen in two rats. In one rat, some macrophages were observed perivascularly and peribronchially. At a 0.8 mg/kg inulin dose, a few polymorphous nuclear cells were found in one rat in the alveoli and perivascularly, while in two rats alveolar macrophages were found.

In the control group that received insufflation without powder, a single small infiltrate of polymorphous nuclear cells in the interstitium and peribronchially was observed in a single rat. In addition, a few alveolar macrophages were found in another rat (Figure 3). Control rats that did not receive insufflation but were treated according to the same experimental procedures, showed a similar picture as the insufflation group (Table 2).

Table 2. Semiquantitative histologic observations in the experimental groups

Group	Treatment	Cell types	Interstitium	Vascular	Central	Bronchial	Alveo-lar
1	5 mg/kg iCsA	Neutrophils	++	+/-	+/-	+/-	++
		Macrophages	+++	+/-	++	++	++
		Lymphocytes	+++	+/-	++	++	++
2	0.8 mg/kg iCsA	Neutrophils	+	+/-	-	-	-
		Macrophages	+	-	-	-	+/-
		Lymphocytes	-	-	-	-	-
3	5 mg/kg placebo	Neutrophils	-	++	-	+/-	-
		Macrophages	-	+/-	-	-	-
		Lymphocytes	-	-	-	-	-
4	0.8 mg/kg placebo	Neutrophils	-	+/-	-	-	+/-
		Macrophages	-	-	-	-	+
		Lymphocytes	-	-	-	-	-
5	Insufflation	Neutrophils	+/-	-	-	+/-	-
		Macrophages	-	-	-	-	+/-
		Lymphocytes	-	-	-	-	-
6	-	Neutrophils	-	+	-	+/-	-
		Macrophages	-	-	-	-	-
		Lymphocytes	-	-	-	-	-

Note: The signs denote in how many rats in the experimental a certain cell type was observed: - correlates to no observations, +/- correlates to an observation in 1 rat, + correlates to 2, ++ correlates to 3 and +++ correlates to observations in all rats of a specific group.

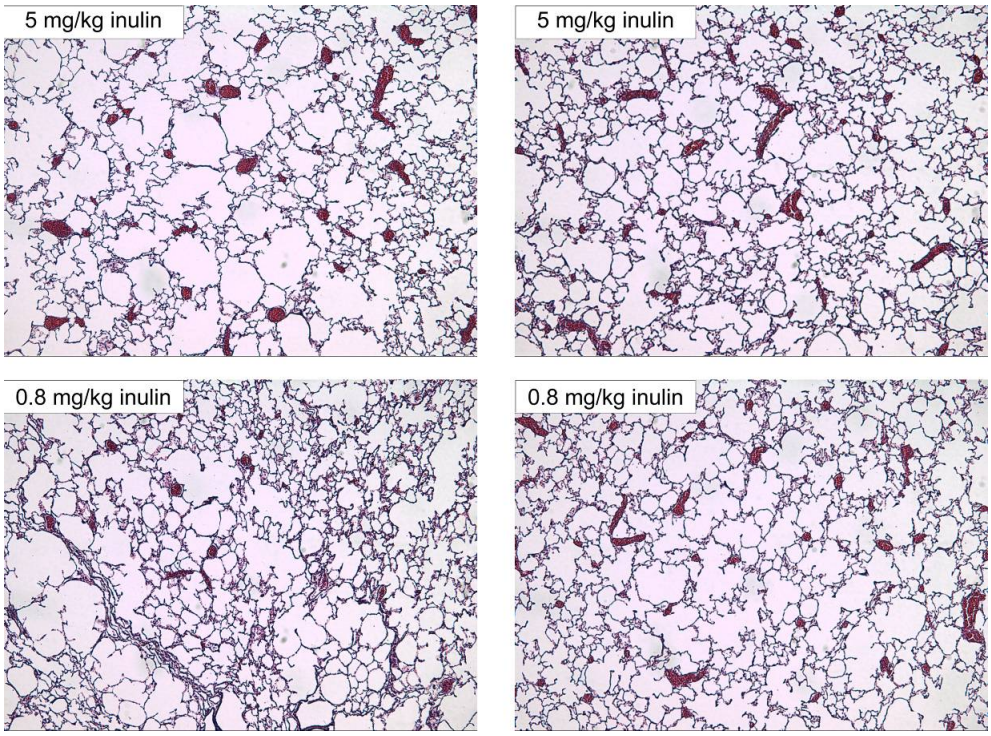

Figure 2. Hematoxillin and eosin stainings of lung tissue from rats treated with placebo (inulin) in a 5 mg/kg (upper part) and 0.8 mg/kg dose (lower part). Each representative photograph is taken from the lung of a different rat. Magnification was 5x.

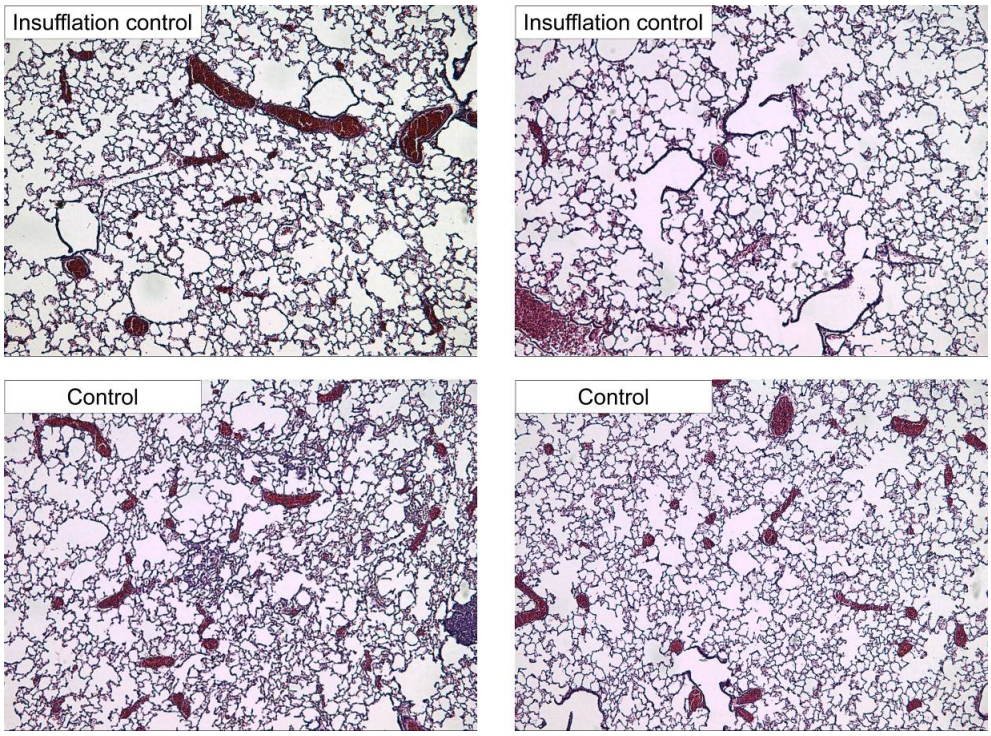

Figure 3. Hematoxillin and eosin stainings of lung tissue from control rats treated with insufflation without powder (upper part) and untreated rats (lower part). Each representative photograph is taken from the lung of a different rat. Magnification was 5x.

DISCUSSION

iCsA in a low dose (0.8 mg/kg) does not result in a local pulmonary reaction as observed after 5 mg/kg inhalation. The reaction after 0.8 mg/kg iCsA was not different from that observed after 5 or 0.8 mg/kg placebo. Furthermore, the reaction observed with 0.8 mg/kg iCsA does not differ essentially from the untreated, but insufflated control groups. As a summary measure, the cumulative number of histologic abnormalities ranked as follows: 5 mg/kg iCsA (27 abnormalities) >> 0.8 mg/kg iCsA (6 abnormalities) > 5 or 0.8 mg/kg inulin (4 abnormalities) > insufflation or placebo (3 abnormalities). Additionally, the intensity of observed histologic reaction was much less intense than the reaction caused by insufflation of 5 mg/kg iCsA, indicating that the lower iCsA dose is safe. Inulin insufflcation appeared to be safe at both doses.

Our results suggest that the high CsA levels locally in the lung after 5 mg/kg iCsA are the main reason for the observed pulmonary reaction. In the previous study (7), only the 5 mg/kg dose was effective in preventing allograft rejection while a lower dose did not inhibit rejection. Therefore, demonstrating that 0.8 mg/kg is safe may seem irrelevant. Nevertheless, the animal model used in the allograft rejection study was very acute because of the incompatibility between the major histocompatibility complexes of the donor and recipient. This incompatibility necessitated the use of high iCsA doses, administered via the pulmonary route as single treatment, for effectiveness. In contrast, in the human transplantation setting, the donor and recipient are matched to prevent acute rejection as much as possible. Consequently, immunosuppresive therapy is given for a long time via the oral route, with a combination of immunosuppressive drugs. If iCsA was to be used in humans then iCsA would be given on top off the current treatment regimen, and never as a single treatment. For that situation, the 0.8 mg/kg dose administered in the rat will probably be too high as the 0.8 mg/kg iCsA dose in rats corresponds to 64 mg CsA given by inhalation in humans. In the currently scheduled clinical trial, we envisage to use a dose of 6.25 mg CsA, equivalent to 12.5 mg powder. This dose is based on a study performed by Corcoran *et. al.* (9), who reported that for nebulized CsA, a deposition of at least 5 mg in the peripheral lung led to improvements in lung function after lung transplantation. The 6.25 mg CsA dose is based on our intention to administer iCsA twice daily and the assumption that we will be able to obtain about 40% deep lung deposition with this powder and administration device (Twincer®). Adverse side effects as seen with the 5 mg/kg dose in the rat are not expected with the 6.25 mg iCsA human dose since the human dose is over 60 times lower (and even 10 times lower than the 0.8 mg/kg rat dose tested in this study).

In the previous study we concluded that in humans inhalation of iCsA as a single treatment would not be feasible while a lower iCsA dose in combination with systemic immunosuppression could have a place in therapy. As shown in this study, a lower iCsA dose led to a reaction at the level of placebo. More important, the similarity in reaction between 0.8 mg/kg iCsA, placebo and controls suggests that the observed effects at this dose are merely the result of the experimental setup rather than the administered drug.

To conclude, the results described in this chapter demonstrate that a 0.8 mg/kg dose of iCsA does not give the pulmonary adverse reaction found with a 5 mg/kg dose (7). This indicates that there are no major safety objections against the use of this 0.8 mg/kg dose in man. Although in the previous study a lower dose of inhaled CsA was not effective in suppressing rejection in a very acute animal model of rejection, a study that combines systemic (oral) with pulmonary CsA administration may demonstrate the benefits of a cyclosporine powder for inhalation while preventing at least large part of the associated nefrotoxicity.

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CHAPTER 5

The preclinical development of (recombinant human) deoxyribonuclease I as a powder for inhalation

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ABSTRACT

A formulation and process development study was performed to formulate recombinant human deoxyribonuclease I as a powder for inhalation. First, excipient compatibility (with bovine DNase as a model substance) was examined with a stability study at stressed conditions (60 and 85 °C) while monitoring for occurrence of the Maillard reaction. Next, powders for inhalation were prepared by spray drying and spray freeze drying. We found that spray drying with inulin as stabilizer resulted in the best powder for inhalation. Finally, an ex-vivo test with the spray dried rhDNase I/inulin powder significantly decreased elastic and viscous moduli of sputum from 5 cystic fibrosis patients.

INTRODUCTION

Pulmozyme® is a solution for nebulization containing 2.5 mg/mL recombinant human deoxyribonuclease I (rhDNase I). RhDNase is used to decrease the viscoelastic properties of sputum in cystic fibrosis (CF) patients which results in an improved lung function (1, 2). In CF, repeated respiratory tract infections lead to high concentrations of DNA from degenerating polymorphonuclear leucocytes in the lung (3, 4). The presence of DNA increases the viscosity of sputum and makes it more difficult to clear the sputum from the respiratory tract. This increases the susceptibility to infection which, in turn, provokes an inflammatory response. The cycle of infection, inflammation and obstruction leads to progressive destruction of lung tissue and reduced life expectancy (5, 6).

Pulmozyme is one of the therapeutic options in CF. In a double-blind, randomized, placebo-controlled phase III study, which involved 51 CF centers and 968 CF patients, patients were randomized to receive 2.5mg rhDNase once or twice daily, for 6 months (7). Administration of rhDNase either once or twice daily reduced the risk of a respiratory infection requiring antibiotics by 22% and 34%, respectively. At the end of the 6-month period the improvement in forced expiratory volume in 1 second (FEV1) was 0% for the placebo group, 5.8% for the once-daily group, and 5.6% for the twice-daily group, showing that both the incidence of respiratory tract infections and lung function is improved by rhDNase.

Pulmonary administration of drugs by nebulization is a process with several drawbacks. One of the major drawbacks is low deposition efficiency, especially with traditional nebulizers which for instance have high residual volumes. For Pulmozyme, the pulmonary deposition was reported to range between 7.8% and 31.2% (8). Furthermore, the nebulization (including cleaning of the equipment) is time consuming and requires complex equipment using electricity or compressed air. Finally, the solution for nebulization has to be stored in the refrigerator (2-8°C). All these aspects reduce patient compliance to therapy and may reduce the success of rhDNase therapy. The development of a stable dry powder inhalation formulation containing rhDNase may improve the administration. Major advantages are the potentially higher lung deposition (which would also reduce costs), a reduced administration time and no need to store the rhDNase solution at low temperatures to prevent degradation (8-12). These advantages may results in an improved compliance which positively affects the therapeutic outcome.

Major aspects that have to be taken into account when a protein containing dry powder for inhalation is to be developed are:

- the excipients in the formulation should stabilize the protein during drying and subsequent storage,
- the powder characteristics should, in combination with the used inhalation device, allow for the generation of an aerosol with an aerodynamic particle diameter within the 1 to 5 µm range (preferably 2 µm) (13).
- ex vivo experiment should guarantee the activity of the protein in the therapeutic setting.

Drying of pure proteins often leads to partial or total inactivation due to degradation. However, recently several technologies have been described that allow for the production of a dry and stable powder formulation of proteins for inhalation (14-17). To withstand the degradation during processing and storage, stabilizing excipients should be used (18-21). The most common types of protectants are carbohydrates or polyols (e.g., mannitol, sucrose, trehalose, inulin). Spray (freeze) drying of aqueous solutions of rhDNase has been reported previously as a successful drying technique (14, 16). Maa *et al.*(16) have compared spray drying with spray freeze drying and found that with their inhaler (Dryhaler®) spray freeze drying was better suited to produce a rhDNase powder for inhalation. However, their findings may be rather specific for the used inhaler and spray freeze drying, in contrast to the spray drying, is difficult to upscale to an industrial process (22).

Quan *et al.* (23) have demonstrated that rhDNase is prone to the Maillard reaction when intimately co-formulated with reducing sugars, such as lactose. Hence, reducing sugars should be avoided as excipient for proteins in spray dried and freeze dried formulations. In a different study, Chan *et al.* (14) have spray dried pure rhDNase solutions and subsequently mixed the powder with lactose carrier, thereby keeping the number contact points between rhDNase and lactose low. However, for long term storage, a glassy formulation may be preferred.

The purpose of this study was to investigate whether spray drying of a (rh)DNase powder for inhalation with the Novolizer® dry powder inhaler is feasible. In this study the performance of spray dried DNase powder is evaluated in comparison with freeze dried and spray freeze dried powders regarding stabilization effectiveness, maintenance of enzymatic activity and dimerization of (rh)DNase. In contrast with previous studies, the

aerosol generation of the differently produced powders was studied without mixing the powders with additional excipients (e.g. crystalline lactose) to improve dispersion. Working without additional excipients reduces the amount of material to be inhaled. Finally, an ex vivo test, measuring the effect of rhDNase powder on the viscoelasticity of CF sputum, was performed.

MATERIALS AND METHODS

Materials

Pancreatic bovine deoxyribonuclease I (DNase, ~3000 units per mg, purity >80%) was purchased from Sigma-Aldrich. DNase was used as a model drug, since bovine DNase has a homology of ~80% in its amino acid sequence compared to rhDNase (24). DNase was the test drug for the entire study, with the exception of the ex vivo proof of principle, where recombinant human deoxyribonuclease I (rhDNase) was used, which was obtained as Pulmozyme® from Roche (Basel, Switzerland). Inulins with a degree of polymerization (DP) of 14 and 23 were a generous gift of Sensus (Roosendaal, the Netherlands). All other chemicals were either of reagent or of analytical grade and purchased from commercial suppliers.

Methods

Powder preparation methods

For all processes used to produce bovine DNase powders, the starting solutions were made in the same way. Each solution contained 2.5 mg/ml DNase, 0.375 mg/ml $CaCl_2$ and 11.0 mg/ml NaCl and the pH was adjusted to 6.3 with 0.2N HCl. $CaCl_2$ was added to the DNase-solution because it has been reported that this salt stabilizes two disulfide bridges in the protein (23). NaCl was added to ensure that DNase remained in its tertiary structure in the solution. Carbohydrates were added to the DNase solution in a final concentration of 22.5 mg/ml before drying.

Spray drying

Sucrose, trehalose, inulin DP14 and inulin DP 23 were initially tried in the spray drying process. For the inhaler experiments inulin DP23 was used as stabilizer. The solution was spray dried with a Büchi 190 Mini Spray Dryer (Büchi, Flawil, Switzerland) equipped with a two-fluid nozzle (0.5 mm). The process conditions were: pump 2.37 mL/min (setting 3), aspirator 75 mbar (setting 18), heating inlet temperature 135 °C, outlet

temperature between 55 - 75 °C (setting 9) and the atomizing airflow was set at 400, 600 or 800 L_n/h. The obtained powder was subsequently stored at 20 °C and 50% relative humidity (RH).
To spray dry rhDNase, Pulmozyme® was used as starting material and the preparation of the solution was adapted to obtain similar concentrations as during DNase experiments. Firstly, Pulmozyme® was centrifuged with an Ultrafree© centrifugal filter device with a molecular weight cut-off of 10 kD (Millipore Corporation, Bedford, MA 01730) to obtain a more concentrated solution. After centrifugation, the concentration of rhDNase was measured with a Lowry assay (25). The $CaCl_2$ concentration was adjusted to the rhDNase concentration with microdialysis using a pH of 6.3. Afterwards, inulin DP23 was added to obtain a ratio of rhDNase:inulin of 1:9. The total concentration of inulin and rhDNase was 40 mg/mL and contained 0.6 mg/mL $CaCl_2$ at a pH of 6.3. The solution prepared in this way was similar to the solutions used for bovine DNase, only the concentration was slightly higher because centrifugation led to higher rhDNase concentrations. A similar solution was prepared without rhDNase to obtain a placebo powder.

Freeze drying
Sucrose, trehalose, inulin DP14 and DP 23 were used as stabilizers in the freeze drying process; a sample without carbohydrate was produced as reference material. The sample without carbohydrate thus only contained DNase (2.5 mg/ml), $CaCl_2$ (0.375 mg/ml) and NaCl (11.0 mg/ml). Freeze drying was performed using a Christ model Alpha 2-4 lyophilizer (Salm en Kipp, Breukelen, the Netherlands). For the freeze drying experiments, 4 mL glass vials were filled with 400 µl aqueous solution. The aqueous solutions were frozen in liquid nitrogen and subsequently dried at a condenser temperature of -53 ºC and a shelf temperature of -35 ºC at a pressure of 0.220 mbar. After 21 hours the shelf temperature was increased incrementally over 3 hours to 20 ºC and the pressure was decreased to 0.060 mbar. Secondary drying was continued for another 24 hours under these conditions.
After lyophilization the dried samples were transferred to a vacuum desiccator and stored for at least 1 day before using the samples in experiments.

Spray freeze drying

For the spray freeze drying process (with inulin DP23) the solution was sprayed into a bowl of liquid nitrogen with the two-fluid nozzle (diameter 0.5 mm). The same nozzle as used in the Büchi Mini Spray Dryer. The nozzle was placed approximately 10 cm above the surface of the liquid nitrogen and the solution was atomized with an atomizing air flows of 400, 600 and 800 L_n/h at a pump flow 2.37 mL/min. After the spraying was completed, the bowl with liquid nitrogen was transferred to a Christ model Alpha 2-4 freeze dryer (Salm & Kipp, Breukelen, The Netherlands) and the liquid nitrogen was allowed to evaporate. For drying, exactly the same regime was used as described for the freeze dried samples The obtained powders were subsequently stored at 20 °C and 50% RH.

Stability testing of the powders

Freeze dried samples were transferred to a vacuum desiccator for at least one day before using the samples in experiments. Long term storage of the freeze dried samples was at 0% RH and two different temperatures: 60 and 85 °C. The climates were controlled with a Hygrocontrol temperature and humidity transmitter (Hygrocontrol, Hanau, Germany).

DNase activity

The effect of freeze drying in the absence or presence of carbohydrates followed by storage at elevated temperatures on the enzymatical activity of DNase was determined as described by Sinicropi (26). Samples were reconstituted with 2.5 mL of HEPES buffer (containing 25 mM HEPES, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 0.1% BSA, 0.01% thimerosal and 0.05% Tween 20) and diluted to DNase concentrations of 2000 ng/mL, 1000 ng/mL and 500 ng/mL. A calibration curve of unprocessed DNase was simultaneously prepared in HEPES buffer with a concentration range of 0-1000 ng/mL. The substrate was prepared by mixing 8.47 mL buffer A (25 mM Hepes and 1 mM EDTA, pH 7.5) with DNA from salmon testes (Sigma), 0.42 mL buffer B (20 mM acetate-NaOH, pH 4.2) containing 0.4% methylgreen and 2.21 mL buffer C (25 mM Hepes, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 0,1% BSA, 0,01% thimerosal, 0,05% Tween 20, pH 7.5).

Next, the wells of a 96 wells plate were filled with 100 µl of the diluted sample and 100 µL substrate was added. The reaction was performed at room temperature. After 90 minutes the reaction was quenched by adding 50 µL of a solution containing 50 mM EDTA and 50 mM H_2O_2. After another

90 minutes the absorption was measured with a Biorad Microplate Reader Benchmark (Biorad, Hercules, United States) at 630 nm. The difference in absorption between 5 and 180 minutes was used to calculate the activity of the samples relative to unprocessed DNase.

Glass transition temperature

The glass transition temperature of freeze dried samples was determined with modulated differential scanning calorymetry (mDSC; DSC2920 differential scanning calorimeter, TA Instruments, Ghent, Belgium). The sample was preheated at 40 °C for 15 minutes and then heated to 200 °C at a rate of 2 °C/min and a modulation of ±0.318 °C/min. The midpoint of the deflection in the reversing heat flow – temperature curve was determined as the T_g. During the measurements, the cell was purged with nitrogen (35 mL/min).

Reducing groups

The amount of reducing groups in the carbohydrates was determined with the Sumner assay (27). In short, 100 mL of a solution with 20 g NaK-tartrate tetrahydrate, 1 g dinitrosalycylic acid, 1 g NaOH and 200 mg phenol was prepared. Subsequently, 1.2 mL of this solution was added to 0.8 mL of the carbohydrate solution. To this solution, 80 µL of a freshly prepared solution of 3 g Na_2SO_3 in 100 mL demineralised water was added. The resulting mixture was immediately vortexed and placed in a water bath of 95 °C. After 15 minutes the samples were removed from the water bath and allowed to cool down to room temperature. Finally, the absorbance of 200 µL was measured at 630 nm using a Biorad Microplate Reader Benchmark (Biorad, Hercules, United States). A calibration curve of glucose in a concentration range of 0.09 – 0.9 mg/mL was used in all experiments. The number of reducing groups was expressed as the percentage of sugar units containing reducing groups.

Monitoring of the Maillard reaction

The Maillard reaction occurs in three steps. In the first step the reaction between the carbonyl and free amino groups leads to a condensation product N-substituted glycosylamine, which transforms into the Amadori rearrangement product (ARP). Under acidic conditions, the ARP undergoes mainly 1,2-enolisation with the formation of furfural (when pentoses are involved) or 5-hydroxy-2-methylfurfural (when hexoses are involved). In

the intermediate step colorless Maillard intermediates are formed, such as 5-hydroxymethylfurfural and pyrazines. In the last step of the Maillard reaction the advanced glycation end-products are formed which can be noticed by their brown color (nonenzymatic browning) (28). The formation of 5-hydroxymethylfurfural and pyrazynes was investigated by measuring the absorbance of reconstituted samples at 280 nm using a Unicam UV 500 UV-VIS spectrophotometer (Thermospectronic, Cambridge, UK) (29, 30).

Dimeric aggregation

Size exclusion chromatography was performed on all DNase-carbohydrate samples stored for 0, 6, 14, 21 and 28 days at 85 °C. Prior to analysis, samples were dissolved with 1.0 mL HEPES buffer (as described under DNase activity) and filtered using a 0.45 µm filter. Subsequently, all solutions were placed in a Perkin-Elmer 200 series equipped with a Bio-Sil SEC250 column and a Bio-Sil 250 guard. As mobile phase 0.1 M sodium phosphate and 0.1 M sodium sulphate with a pH of 6.8 was used at a flow rate of 1.0 mL/min. For each run 20 µL sample solution was injected . Detection was performed with an ultraviolet detector (Applied Biosystems model 785A) at 215 nm. For calibration a GPC standard (Bio-Rad Laboratories, Hercules, USA) was used which contained bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa) and vitamin B_{12} (1.35 kDa). The molecular weight of DNase is 31 kDa.

Scanning electron microscopy

Scanning electron micrographs (SEM) were recorded with a JEOL JSM 6301-F Microscope (JEOL, Japan). The powder was dispersed on top of double-sided sticky carbon tape on metal disks and coated with 150 nm of gold/palladium in a Balzers 120B sputtering device (Balzers UNION, Liechtenstein).

Laser diffraction analysis

Particle size distributions of the powders were measured in duplicate (the difference between the duplicates was less than $\pm$1% for all samples) with a HELOS Compact model KA laser diffraction apparatus (Sympatec GmbH, Clausthal-Zellerfeld, Germany: 100 mm lens and the Fraunhofer theory)

equipped with a RODOS disperser. The powders were dispersed at a pressure of 3 bar and the median volume diameters (X_{50}) were calculated.

Cascade impactor analysis

Cascade impactor analysis was carried out with a glass constructed four stage liquid impactor of the Fisons type (Elgebe, Leek, The Netherlands). The Novolizer® inhaler was attached to a dry bent induction port using a coupling flange with rubber seal ring. A solenoid valve was used in combination with a timer to control the flow rate through the inhaler and the cascade impactor for the duration of 3 seconds. The airflow through the inhaler, corresponding with a pressure difference of 4 kPa, was 71 L_n/min. The amount of drug deposited on the different stages was determined by the Lowry assay (25). Deposition was expressed as a percentage of the metered dose. The fine particle fraction (FPF), defined as a percentage of particles with an aerodynamic diameter less than 5 µm, was calculated by intrapolation of the cumulative mass plot versus effective cut-off diameter and expressed as a percentage of the metered dose. The mass median aerodynamic diameters (mmad's), were calculated from the cumulative mass percentages derived from the impactor stages 2, 3 and 4 (31), using the theoretical cutpoints for the different impactor stages.

Cascade impactor measurements were performed in triplicate.

Ex vivo rhDNase activity analysis

Approval for the collection of sputum of CF patients was obtained from the ethics committee of the University Hospital of Ghent. The sputum was expectorated spontaneously by CF patients (n=5) during chest physiotherapy sessions. After collection, sputum samples were immediately frozen at -20 °C (32, 33). The prepared placebo and rhDNase powder was mixed in a 1:10 ratio with sucrose to obtain a weighable dose of rhDNase. In total, rhDNase-dose was 30 µg (15 IU).

After thawing, a single sample of sputum was divided into 4-6 portions, depending on the volume of the sample. First, the viscoelasticity of untreated sputum was determined as 100% reference. Next, 30 mg of placebo powder or rhDNase powder was added and the viscoelasticity of the sputum was determined after 20 minutes of incubation at room temperature. This procedure was performed in such a way that at least

duplicate measurements were determined on a single sputum sample of placebo and DNase powder.
Sputum viscoelasticity was measured with a controlled-stress rheometer (AR 1000-N; TAInstruments, Ghent, Belgium) at 20 °C, using a cone-plate geometry. The angle between the cone and the plate was 2 degrees, and the sample volume required was approximately 0.9 mL. Dynamic oscillatory measurements were made at a constant frequency of 1 Hz, with a stress ranging from 0.01 to 0.1 Pa. To avoid disruption of the weak biopolymer network in sputum, the elastic (G') and viscous (G") moduli of the sputum samples were determined in the linear viscoelastic region.

RESULTS

Excipient compatibility and stability

To determine the effect of different excipients on the stability of bovine DNase the material was freeze dried in the presence of the four different carbohydrates. Figure 1 shows that freeze drying reduces the activity of bovine DNase by approximately 20% when no carbohydrate is present in the formulation. Drying in the presence of a carbohydrate reduces the activity loss to about 10%.
When stored at 60 °C all carbohydrates protected DNase from further loss of activity for a period of 6 weeks, whereas the DNase freeze dried without carbohydrate lost another 40% of activity within the same period. The type of carbohydrates did not seem to have a substantial effect at this storage temperature.

Table 1. Glass transition temperature and the percentage of reducing groups found for the different carbohydrates (freshly prepared freeze dried samples, n=3).

Sample	T_g (°C)	Reducing groups (%)
Sucrose	76.1 ± 1.1	0.09 ± 0.01
Trehalose *	121.6 ± 0.7	0.14 ± 0.04
Inulin DP14 *	139.7 ± 1.7	2.1 ± 0.1
Inulin DP23	157.1 ± 1.3	5.2 ± 0.2

* Data from Hinrichs *et al.* [21]

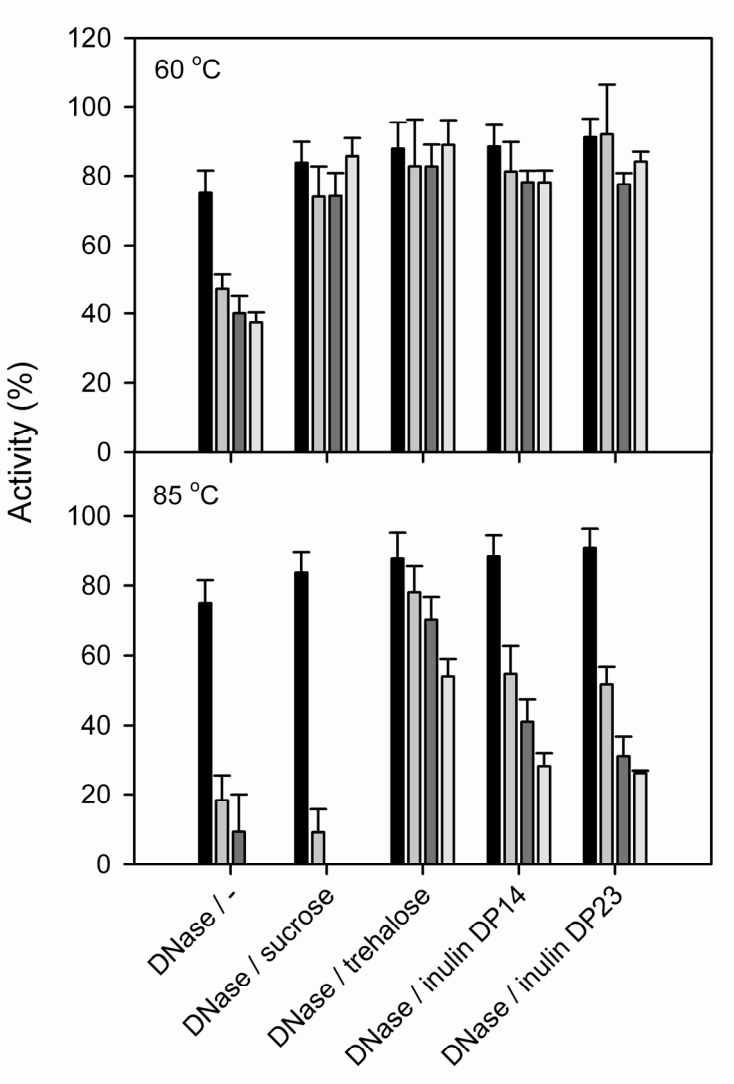

Figure 1. DNase activity of the different formulations after freeze-drying and storage at 60 °C or 85 °C. Storage times were 0 days (black bars), 2 weeks (grey bars), 4 weeks (dark grey bars) or 6 weeks (light grey bars). Results are expressed as averages (n=3) ± standard deviation.

When stored at 85 °C, DNase freeze dried without carbohydrates rapidly lost all of its activity. The sucrose formulation lost its activity even faster under these conditions. However the degradation rates of the trehalose and inulin formulations were clearly reduced compared to the reference

and the sucrose formulation. The formulations with inulin showed a faster loss of activity than the trehalose formulation, despite the higher Tg's of inulin (Table 1). In a control experiment, the presence of NaCl in the formulations did not change the T_g of the pure carbohydrate (data not shown). Visual observation of the stored cakes showed a dramatic volume contraction and browning, which was dependent on storage time. This effect was most pronounced for the sucrose-containing formulation stored at 85 °C. At this temperature, the sucrose formulation was stored above its Tg.

The Sumner assay revealed that sucrose and trehalose contain only minor levels of reducing groups whereas somewhat higher levels were found in the inulins (Table 1).

The intrinsic absorbance of DNase at 280 nm in all formulations immediately after freeze drying (t = 0) was found to be 0.18 (Figure 2A). During storage at 85 °C, the absorbance increased dramatically for the sucrose formulation, indicating a dramatic increase in the formation of 5-hydroxymethylfurfural and pyrazines (intermediates of the Maillard reaction). The increase was much less pronounced for both inulins, whereas the trehalose formulation showed no significant increase in absorption at 280 nm.

Upon reconstitution, approximately 10% of DNase was found as dimeric aggregates immediately after freeze drying, irrespective of the formulation (Figure 2B). During storage at 85 °C, the sucrose formulation resulted in the highest increase in the amount of dimeric aggregates. Also, the inulin formulations and the carbohydrate-free DNase showed a slight increase in the amount of dimeric aggregates during storage whereas this increase for the trehalose formulation was almost negligible.

Drying processes

In this study spray drying was chosen as process of first choice for the production of a dry DNase powder for inhalation. However, when spray drying was performed it was found that the sucrose formulation can not be processed properly at the conditions tested in our study. The particles immediately coalesced in the collection vessel. The trehalose and inulin formulations could successfully be spray dried but the trehalose formulation could not be stored adequately at ambient conditions (20-25 °C and 40-60% RH), due to coalescence of the particles, rendering an unsuitable formulation for inhalation. The inulin formulations showed

no coalescence during storage at ambient conditions, but in further spray drying experiments, we decided to investigate only the inulin DP23 formulation. The enzymatic activity of both the bovine DNase as well as of the rhDNase was found to be around 80% after spray drying.

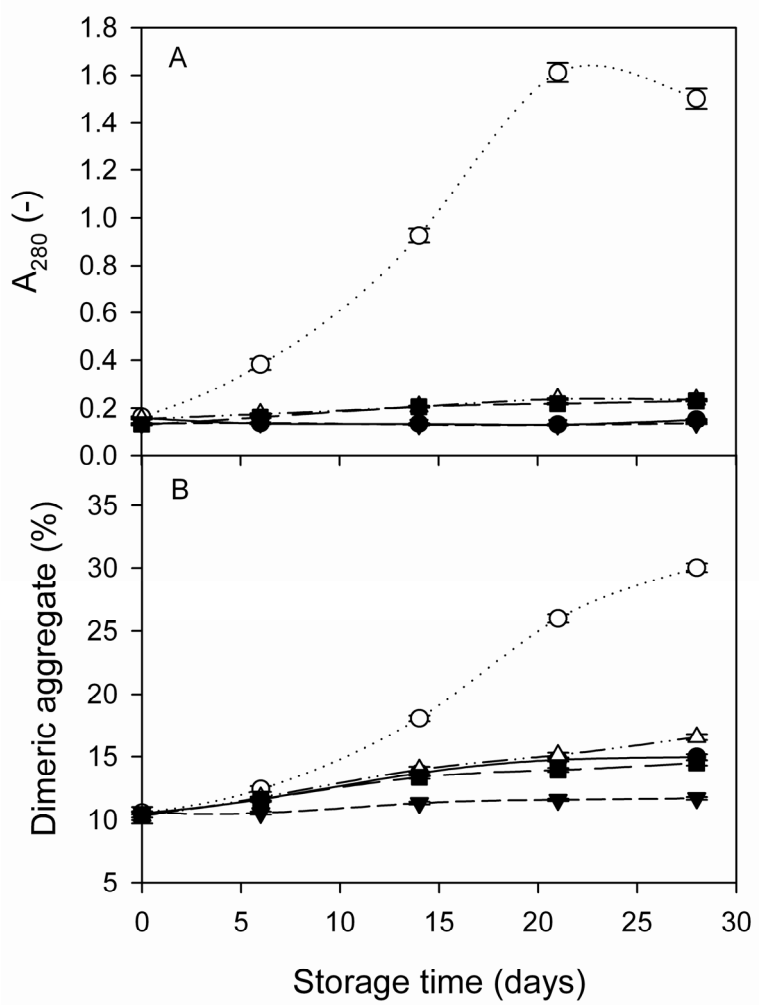

Figure 2. A: UV absorption at 280 nm of reconsituted freeze dried formulations after storage at 85 °C. B: percentage dimeric aggregates as found with size exclusion chromatography of reconstituted freeze dried formulations after storage at 85 °C: pure DNase (●), DNase-sucrose (○), DNase-trehalose (▼), DNase-inulin DP14 (■) and DNase-inulin DP 23 (△). Results are expressed as averages (n=3), error bars represent standard deviation.

Because inulin was the only carbohydrate to be processed successfully with spray drying, inulin (only DP23) was also selected for the preparation of a spray freeze dried powder in order to investigate the effect of process (spray drying vs. spray freeze drying) on the aerosol formation properties in inhalation experiments.

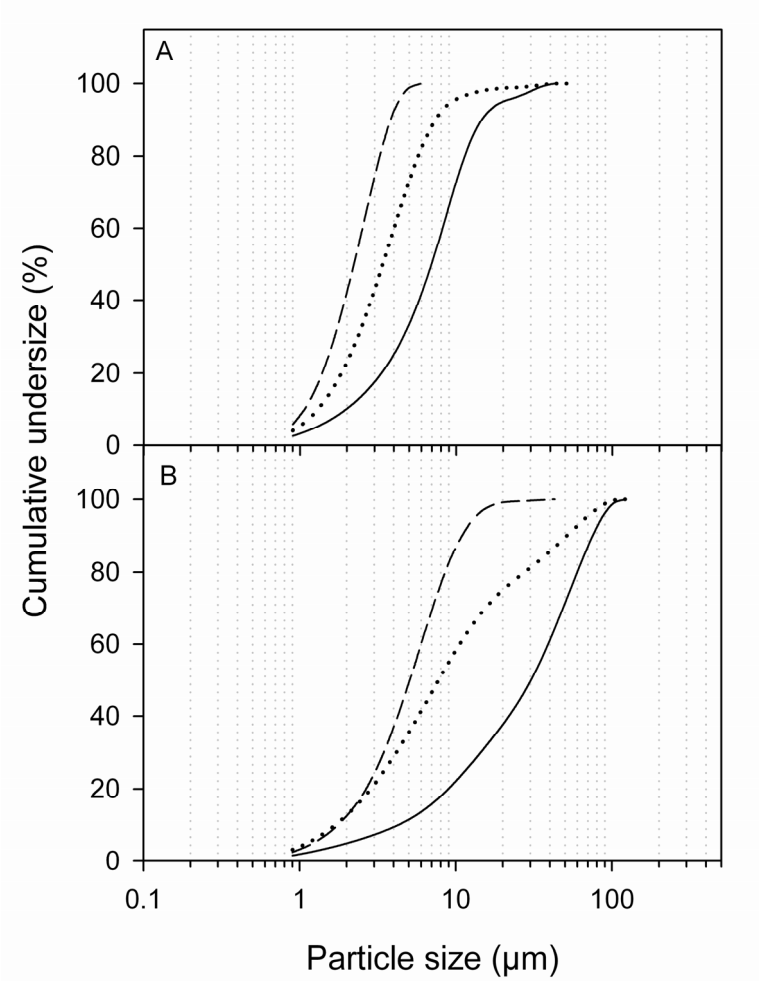

Figure 3. Effect of atomizing air flow on the powder particle size distribution of the DNase-inulin DP23 formulation after spray drying (A) or spray freeze drying (B) as determined by laser diffraction. Solid line represents 400 L_n/h, dotted line 600 L_n/h and crossed line 800 L_n/h.

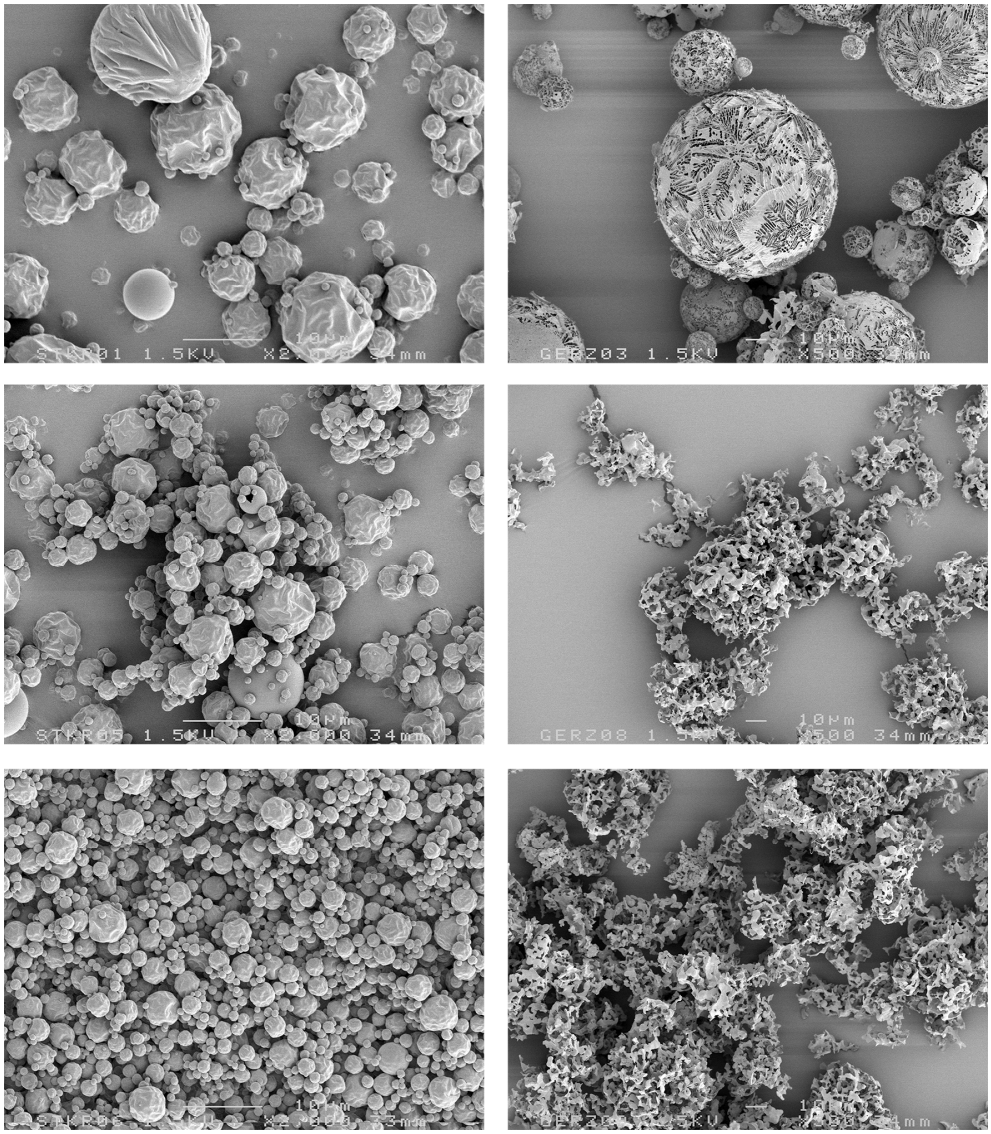

Figure 4. Scanning electron micrographs of spray dried (left) or spray freeze dried (right) of the DNase-inulin DP23 formulation. From top to bottom: atomizing air 400 L_n/h, 600 L_n/h and 800 L_n/h, respectively. Magnification factor of the spray dried formulation was 2000x and for the spray freeze dried formulation 500x.

Physical characteristics and in vitro deposition of the powders

Laser diffraction experiments revealed that spray drying at a higher atomizing air flow results in smaller particles (Figure 3A). The same effect was also found for spray-freeze drying (Figure 3B). Furthermore, spray freeze drying produced larger particles than spray drying at similar atomizing air flows (see also table 2). The morphologies of the particles obtained by spray drying and spray freeze drying are shown in Figure 4. Spray drying resulted in basically round, slightly dimpled particles. Spray freeze drying resulted in larger particles. At an atomizing air flow of 400 L_n/h, round intact spheres were produced, while at 600 and 800 L_n/h, the particles consisted of fragments of these spheres. These fragments tended to agglomerate and form larger "secondary" particles.

To determine the fine particle fraction from a marketed inhaler (Novolizer®) we performed a series of cascade impactor analyses with the spray dried powders. Figure 5A shows that a significant fraction of all three samples produced at different processing conditions deposited in the inhaler and induction port. Formulations prepared at increasing atomizing air flow showed an increased FPF.

From the cascade impactor data, mass median aerodynamic diameters were calculated (Table 2). Spray drying at higher atomizing air flows resulted in a powder having a smaller aerodynamic diameter (d_a). The mass median aerodynamic diameters of the powders prepared by spray drying and spray freeze drying at 600 L_n/h differed substantially, despite the fact that the same solutions and nozzles were used to produce the powders (Table 2). Yet, the fine particle fractions of the spray dried and spray freeze dried powders were comparable, 39.2 and 41.8%, respectively The difference in terms of dispersion and aerosol behavior between the powders was considerable (Figure 5B). The spray freeze dried powder displayed less deposition in the inhaler and induction port. In the cascade impactor, the spray freeze dried powder deposited mainly on the first and second stage, whereas for the spray dried powder the highest fractions were recovered from the third and fourth stage. The difference in the size distributions within the fine particle fractions is reflected by the mass median aerodynamic diameters, being 2.31 µm for the spray dried and 3.70 µm for the spray freeze dried product (Table 2).

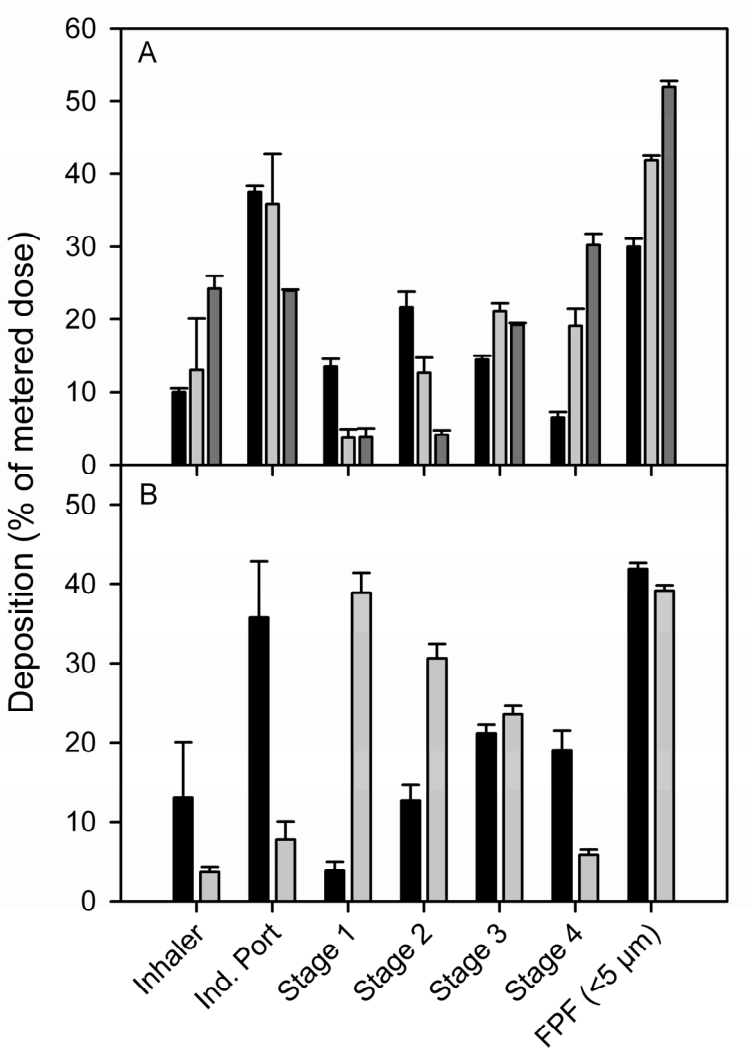

Figure 5. A: Cascade impactor analysis (4 kPa pressure drop) of the DNase-inulin DP23 formulation spray dried at 400 L_n/h (black bars), 600 L_n/h (grey bars) or 800 L_n/h (dark grey bars). B: Cascade impactor analysis (4 kPa pressure drop) of the inulin DP23 formulation spray dried (black bars) or spray freeze dried (grey bars) at 600 L_n/h. Results are expressed as averages (n=3) ± standard deviation.

Table 2. Particle size and aerosol performance of spray (freeze) dried DNase-inulinDP23 as a function of atomizing air flow and. All values are averages of duplicate measurements.

Production process	Atomizing air flow (L_n/h)	x_{50} (µm)	MMAD (µm)	FPF (%)
Spray drying	400	9.15 ± *0.38*	3.57 ± *0.01*	30.0 ± *1.1*
Spray drying	600	7.50 ± *0.92*	2.31 ± *0.10*	41.8 ± *0.7*
Spray drying	800	4.95 ± *0.55*	1.52 ± *0.02*	51.9 ± *0.8*
Spray freeze drying	600	18.67 ± *3.92*	3.70 ± *0.01*	39.2 ± *0.7*

X_{50}: median volume diameter
MMAD: Mass median aerodynamic diameter
FPF: fine particle fraction (< 5 µm)

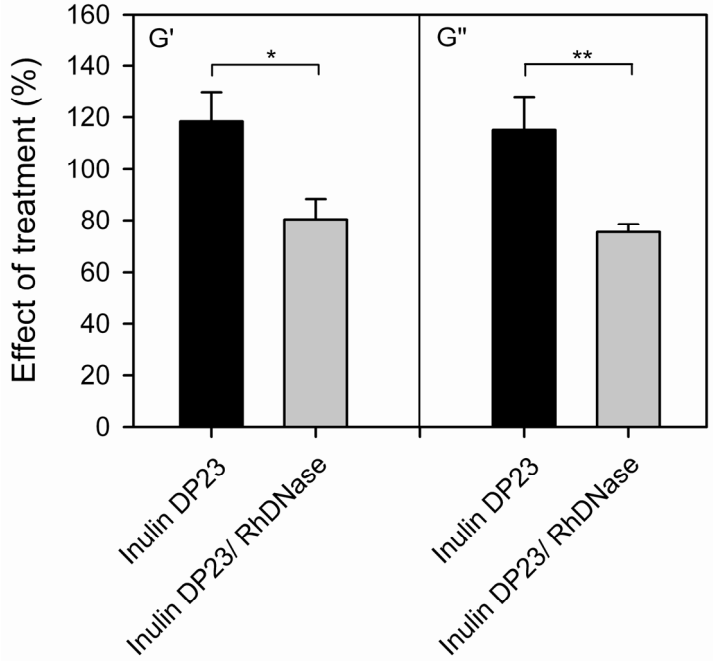

Figure 6. Effect of placebo (inulin DP23) or spray dried rhDNase-inulin DP23 formulation on elastic modulus (G') and viscous modulus (G") of CF sputum. Results are expressed as averages (n= 5) ± standard deviation. Statistics: Effect on elasticity of placebo versus rhDNase-inulin (*, p=0.025), effect on viscosity of placebo versus rhDNase-inulin (**, p=0.016).

Ex vivo rhDNase activity analysis

The effect of a spray dried rhDNase powder on the viscoelastic properties of sputum which was expectorated by 5 CF patients was determined (Figure 6). Addition of placebo powder to the sputum resulted in a 20% increase in elasticity (G') and viscosity (G") compared to the reference measurement. This increase is likely to be caused by partial evaporation of water from the sample due to the length of the measurement. However, addition of powder containing 15 Units rhDNase resulted in a significant decrease of around 40% compared to placebo of both the elasticity (G'; $p=0.025$) and viscosity (G"; $p=0.016$). This indicates that the rhDNase powder dissolved upon contact with the viscous sputum and that the reconstituted rhDNase was able decrease the viscoelasticity of the sputum. For Pulmozyme a similar effect has been described (6, 34).

DISCUSSION

In this study several major aspects related to the preclinical development of a rhDNase containing powder for inhalation were investigated.

Since the spray drying of the sucrose powders was not successful and trehalose rendered powders that showed rapid coalescence at ambient conditions, spray drying was not considered a suitable preparation method for the stability testing. Freeze drying is a suitable alternative since this process, like spray drying, produces powders in which contact between the protein and carbohydrate exists on a molecular scale. Moreover, it was found in this study that after both spray drying and freeze drying (for trehalose and inulin) DNase activity is still around 80%. To compare the effects of the four carbohydrates on the stability of DNase during drying and storage, freeze dried materials were stored under accelerated stress conditions. Fig. 2 shows that all four carbohydrates are suitable stabilizers for DNase at 60 °C. However, at the higher stress condition of 85 °C differences between the carbohydrates are found. The sucrose formulation led to extreme Maillard reaction when stored at 85 °C. This is explained by the fact that at 85 °C the sucrose system is in the rubbery state and the combination with the elevated temperature could have led to the formation of reducing degradation products which subsequently reacted with DNase. On the other hand, for the trehalose formulation no indication of the occurrence of a Maillard reaction was observed. Neither did the absorption at 280 nm increase nor did the amount of dimeric aggregates increase. The degradation found at

85 °C is only heat induced degradation of the DNase. The absence of any interaction of the DNase with trehalose is explained by the high glass transition temperature of the trehalose and the absence of reducing sugars in this material. Both inulin based products show some degradation at 85 °C. Although the degradation rates were considerable slower than in the sucrose product, they were somewhat faster than in the trehalose product. The small increase in the 280 nm absorption and amount of dimeric aggregates shows that the Maillard reaction is to some extent responsible for this degradation. Because of the high glass transition temperature of the inulins (139.7 °C and 157.1 °C for inulin DP14 and inulin DP23, respectively) it is unlikely that reducing degradation products are formed at 85 °C, which indicates that the reducing groups in the starting material are responsible for this degradation. In spite of the higher amount of reducing groups in the inulin DP23 there is no faster degradation compared to inulin DP14. This is explained by the higher glass transition temperature, since for amorphous systems, a low molecular mobility is favorable for storage stability (35).

The spray drying studies revealed another disadvantage of the low glass transition temperature of sucrose. It was found that the sucrose formulation can not be processed properly at the conditions tested in our study. The particles immediately coalesced in the collection vessel, most likely due to a relatively high temperature and residual moisture in the powder by which the Tg was passed. By optimizing the settings, conditions may be found with which the sucrose formulation can well be spray dried, but this was not investigated because of the poor performance of the sucrose in the stability study. The trehalose and inulin formulations could be successfully spray dried. However, the trehalose formulation could not be stored adequately at ambient conditions (20-25 °C and 40-60% RH), due to coalescence of the particles, rendering an unsuitable formulation for inhalation purposes. A similar finding was described by other authors (36). The inulin formulations showed no coalescence during spray drying and subsequent storage at ambient conditions. Based on this observation we decided to perform the further experiments with the inulin DP23 formulation. The inulin DP23 was chosen over the DP14 because of its higher glass transition temperature.

Overall it was concluded that sucrose is not a suitable excipient because of its low glass transition temperature and the formation of reducing degradation products that interacted with the DNase. The high glass

transition temperatures of trehalose and both inulins make these carbohydrates better suited as stabilizer for DNase. Although it would have been preferable that inulin starting materials are free from reducing sugars. On the other hand, the six weeks stability at 60 °C indicates that both trehalose and inulin powders can be stored for more than 1 year at room temperature in the complete absence of water without significant degradation.

During spray freeze drying droplets or fragments of the droplets are rapidly frozen in a rigid conglomerate of solid material and ice. Upon removal of the ice the original droplet dimensions are maintained. With spray drying, water evaporates from the surface of the droplet, resulting in droplet size reduction. At a certain point, a droplet surrounded by a rubbery shell is formed. Upon further drying, the pressure in the particle increases to such an extent that the shell disrupts and often collapses leading to rimpled particles. As a result, the size of the dry particle is often much smaller than the original droplet size. Since the solutions of which the droplets were made contained the same concentration of solid material irrespective of which drying process is used, it is obvious that the spray dried particles must have a higher apparent density. The SEM pictures and laser diffraction results show that an increasing atomizing air flow reduces the particle size when the material is spray dried. The smaller initial droplet size results in a smaller particle size (37).
The large fractions of fragments observed in the SEM pictures of spray freeze dried particles that were produced at atomizing air flows of 600 or 800 L_n/h indicate that the droplets are scattered upon impaction with the liquid nitrogen during the spraying process. Only at the lower atomizing air flow the droplets remain intact when sprayed into the nitrogen.

The cascade impactor experiments clearly show that the reduced particle size of spray dried powders produced with higher atomizing air flows is reflected in an increased fine particle fraction. Also the MMAD decreased for the particles produced at higher atomizing airflows. The particles produced at 400 L_n/h are certainly too large, since Geller *et al*. (13) have reported that nebulization of Pulmozyme in a droplet size distribution with a MMAD of 2.1 µm, resulted in larger improvement of FEV_1 than a droplet size distribution of 4.9 µm: 4.3% vs. 2.5%, respectively. On the other hand, the powder with the MMAD of 1.52 µm may be too small to obtain the desired effects (38). Therefore, the powder produced at 600

L_n/h which gives an MMAD of 2.31 µm from the Novolizer at 4 kPa seems preferable.
The larger MMAD found for the spray freeze dried powder compared to the spray dried powder is remarkable. Since the spray freeze dried powder is formed from the same droplets (or fragments thereof) as the spray dried powder, the same MMAD or a slightly lower MMAD is expected. The higher MMAD indicates that powder de-agglomeration is incomplete for the spray freeze dried powder. This observation is in contrast with the findings of Maa *et al.* (16) who concluded that aerosol performance was superior with spray freeze dried particles. The reason for the better performance of the spray dried particles in this study may be linked to the powder de-agglomeration principle in the Novolizer, which makes use of inertial forces (39, 40). Differences in particle density may cause a difference in classifier payload (at the same dose weight) and this, in combination with a difference in the rate and mode of agglomerate break-up, may result in different effective cutpoints for the classifier. These findings clearly show that drug development for inhalation does not stop at powder formulation, but that inhaler design plays an important role too. Most currently marketed inhalers are not suited for effective de-agglomeration of different types of inhalation powder. Besides, most inhalers do not perform well on powders in the micron range without processing them into adhesive mixtures.

Finally, exposure of the preferred powder formulation to sputum from CF patients resulted in decreased viscosity and elasticity. Sputum from CF patients is extremely viscoelastic because of a low water content and an enhanced presence of extra cellular DNA and actin from degenerating neutrophils, serum proteins, and lipids (4, 41-44). It can be assumed that the mechanism by which the viscoelasticity is decreased by the rhDNase powder is similar to that of the liquid formulation: enzymatic break down of the DNA polymer network and thereby reduction of the typical viscoelasticity of the mucus (2).

In summary, this study shows that a stable rhDNase powder for inhalation can be designed. The stabilizing carbohydrate should be non-reducing and have a high glass transition temperature. Furthermore, utilization of the Novolizer inhaler (which generates high inertial de-agglomeration forces) allowed for the use of the spray drying process to produce the particles

with a high fine particle fraction having a desired MMAD of 2.3 μm. Future research should be directed to patient studies to investigate whether the dry powder inhalation system of rhDNase would indeed increase lung deposition and better fulfill patients' needs in terms of improved ease of administration and compliance and whether this could reduce costs of the therapy (45).

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CHAPTER 6

Pharmaco-economic Review of Recombinant Human DNase (rhDNase) in the management of Cystic Fibrosis; with special reference to current developments in delivery systems

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ABSTRACT

For treatment of patients with cystic fibrosis, recombinant human deoxyribonuclease I (rhDNase) is widely used. RhDNase has a positive effect on the lung function and on the number of hospitalizations. RhDNase is currently administered by nebulization, which is an inefficient administration method. For expensive drugs, such as rhDNase, dry powder inhalation would be advantageous due to increased deposition efficiency, patient mobility and patient compliance. Furthermore, a significant cost reduction might be obtained. We therefore investigated the current status of rhDNase in the management of cystic fibrosis and gave special attention to developments in delivery systems as dry powder inhalation. We preliminarily estimated that if dry powder inhalation of rhDNase could be used, a reduction in the cost-effectiveness ratio of almost 40% can be obtained as compared to nebulization.

INTRODUCTION

Recombinant human DNase (rhDNase) is a mucolytic agent, which reduces the viscoelasticity of sputum and is therefore a possible therapy in cystic fibrosis (CF) patients. RhDNase is currently administered by means of nebulization. However, nebulization is a rather inefficient delivery method. Improved administration of rhDNase would in several aspects be advantageous for CF patients.

Dry powder inhalation (DPI) of rhDNase seems to be a promising example of a more efficient delivery method of rhDNase in CF patients. Because of the high costs of rhDNase, DPI of rhDNase could be cost-saving, compared to the nebulization of rhDNase.

In this review, we evaluate the pharmacoeconomical aspects in the management of rhDNase of CF. Developments in nebulization and DPI delivery systems of rhDNase are compared. Furthermore, aspects with respect to efficacy, tolerability, safety and compliance of rhDNase (nebulization vs. DPI) are outlined.

After weighting the advantages and disadvantages of rhDNase and the delivery systems, an expert opinion and a five-year view with respect to the rhDNase delivery in patients with CF, are described.

CYSTIC FIBROSIS AND RECOMBINANT HUMAN DNASE (RHDNASE)

Epidemiology and Pathofysiology

CF is the most frequently observed genetic disorder in the white population with a potential lethal outcome. CF affects approximately 1 in every 2500-3400 live births and 3.3% is carrier of the defective gene among Caucasians (1-3).

The disease is autosomal recessive, meaning that the gene relating to this disease is not present at the sex chromosome (autosomal) and that a person can carry a defect gene and not have the symptoms but can pass it on to the next generation (recessive). In the Netherlands about 1000 patients have CF, while 1 in every 30 persons carries the defect gene (4). The defective gene is located on chromosome 7. Approximately 70% of the CF cases are caused by a 3 base-pair deletion (ΔF508). The remaining CF cases are arising from about 600 different mutations of the gene (3, 5).

CF is a chronic disease that varies in the underlying systems involved and the severity of the symptoms. This is partly due to the genetic mutations, but also to environmental and treatment factors. In patients with CF

much variability is therefore seen and assessment of the efficacy and effectiveness of treatment strategies takes a long time because of uncertain progression (6).
Complications of CF include pulmonary symptoms, pancreatic insufficiency (in 85% of the patients) leading to malabsorption of fat and malnutrition, diabetes mellitus, biliary cirrhosis, subacute bowel obstructions, arthritis and infertility. Although prevalence is relatively low, the treatment burden per patient may be quite high and can lead to chronic disability at any age (7-9).
Over 85% of the CF patients die from progression of their lung disease (5, 10). Individuals with CF have a mean life span of about 30 years (8). Dietary strategies and new treatments of pulmonary symptoms have led to an increase in average survival of CF patients from only 6 years in the 1960s to about 30 years in the 1990s (11-14). The quality of life for CF patients have also improved dramatically the last decades, due to regular periodic evaluations, monitoring for complications of the disease, and new interventions by physicians and other healthcare workers specifically trained in management of CF (11, 15).
Respiratory Tract Infections (RTIs) are a major cause of hospitalization of CF patients. More than one-third of the CF patients are hospitalized each year, and 20% of the CF patients are hospitalized more than twice a year. Of all CF-related hospitalizations, more than 75% is for treatment of RTIs (16, 17).
The high number of RTIs is a direct consequence of the genetic defect underlying CF. A mutation in the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-dependent chloride channel, causes a defect in the transmembrane chloride transport. This leads to diminished transluminal chloride transport, which results in a relative dehydration of the luminal surface of the exocrine glands. In the lung, the dehydration causes a viscid mucus layer which inhibits a normal ciliary clearing and thus facilitates bacterial colonization of the respiratory tract. Bacterial colonization, most commonly with Staphylococcus aureus, Haemophilus influenza and Pseudomonas aeruginosa, accounts for much of the morbidity and virtually all mortality in CF patients by means of RTIs (1, 2, 7, 8, 16, 18, 19).
After chronic pulmonary infection the viscoelastic properties of purulent airway secretions are primarily caused by DNA from degenerating neutrophils. The presence of DNA increases the viscosity of sputum and

makes it more difficult to clear the sputum from the respiratory tract. This increases the susceptibility to infection which, in turn, provokes an inflammatory response. Therefore, a cycle of infection, inflammation and obstruction leads to progressive destruction of lung tissue and reduced life expectancy (20, 21).
The pulmonary inflammation in patients with CF begins in infancy, is present in babies of about 4 weeks (8), and is probably established when the child is 1 year old (22). The lower respiratory tract of 40% of the children with CF younger than 3 months, has been found to be colonised and in these, as well as in older children, airway DNA levels are also increased (8, 23). This suggests that early inflammation can cause lung injuries, which results in higher susceptibility for inflammation (8).

Pharmacology and Therapy

Patients with CF undergo complex, multidrug treatment aimed on treating the various symptoms of the disease. Alleviating the effects of chronic inflammation and infection in the airways is one of the major objectives. In the last 30 years, airway obstruction reducing agents, airway infection controlling agents, and agents that improve the nutritional status have become the cornerstone of successful treatment in patients with CF (8, 24). Next to these strategies aggressive symptomatic treatment of complications of the lung disease is also important. New treatment strategies are focussed on repair of the basic gene-defect and include manipulation of the ion-transport, activation of mutant CFTR and gene therapy (24, 25).
An important aspect of CF-therapy is the use of mucolytic agents. An example is deoxyribonuclease, which hydrolyzes extracellular DNA and therewith reduces the viscoelasticity of sputum in patients with CF (26). In 1958, bovine pancreatic DNase I was approved in the United States for human use (Dornavac or Dornase). DNase showed a large reduction in viscosity *in vitro* and in one study it was suggested that bovine pancreatic DNase I was reasonably safe and effective in reducing the viscosity of lung secretions (27).
However, for bovine pancreatic DNase I it has been reported that adverse reactions (possibly due to contaminating proteases in the final product) occurred after inhalation, causing the non-human protein to be no longer used (27, 28).

In 1990, recombinant human DNase I (rhDNase) was cloned, sequenced and expressed. RhDNase was *in vitro* shown to dramatically reduce the viscosity of the sputum of cystic fibrosis patients, transforming it within minutes from a viscous non-flowing gel to a flowing liquid (21, 27). Part of the action of rhDNase is explained by cleavage of highly polymerized DNA. Decreasing the chain length of DNA by rhDNase results in a lower viscosity (21). An *in vitro* study showed that the phospholipid concentration (phosphatidylglycerol, dipalmitoylphosphatidylcholine) is increased when the mucus is in its sol phase. The phospholipids are being liberated by rhDNase and then lubricate the mucus, thereby improving the effect of cough and ciliary clearance (28).
In this review, we will focus on the therapy of recombinant human DNase (rhDNase) with special reference to development of delivery systems. Special attention is given to the rational development of RhDNase as a pharmacoeconomically effective drug. In the next section it is motivated that the delivery of rhDNase as a dry powder for inhalation would have enormous pharmaceutical and economical advantages over the current nebulization.

Delivery systems of rhDNase

In order to achieve high local and low systemic concentrations, inhalation is the most advantageous route of administration. Alternative routes of delivery would be very inefficient due to the poor permeation of rhDNase (~ 34 kDa) to airway lumen from the vascular system.
Up to now, rhDNase is administred by means of nebulization. However, maintenance treatment of CF patients with nebulized rhDNase immobilizes the patient to a great extent: there is need for a compressor unit or pressurized air for the jet nebulizer or electricity if an ultrasonic nebulizer is used. Furthermore, the low efficiency is a major drawback. It has been reported that for the jet nebulizers, the type recommended for rhDNase, the efficiency (i.e. the percentage of drug that is available for therapeutic action after the inhalation manoeuvre) is only 2-12% (29). Although rhDNase delivered by nebulization is effective on the short (i.e. 10 days (30, 31)), medium (i.e. 6 weeks (1), 12 weeks (2) and 6 months (32, 33)) and long term (2 years) (34, 35), improved administration would be advantageous for the patient and may have economic benefits. With dry powder inhalation (DPI) there is no need for pressurized air or electricity: the patient delivers the driving force for administering a dose

by means of an inhalation manoeuvre (29, 36). Moreover, DPI has the potential for a more efficient (deep) lung deposition, reports describing efficiency of up to 60% have appeared (37, 38) and new technologies are currently developed (39, 40). A switch from nebulization to DPI is therefore likely to increase patient compliance (dosing time, patient mobility) and therapeutic efficacy (29, 36).
Although the development of a DPI-formulation of proteins is more complex, it is possible to develop such a formulation (41-44). In this review, we examined the consequences of the choice of type of formulation (nebulization vs. DPI) on healthcare costs.

Cost of illness

In the last decennia, new treatment strategies of CF increased median survival of this disease by a factor 5 (8). Cost estimates from developed countries indicate CF as a very expensive disease. Estimates of the annual cost per CF patient vary between €1,954 and €13,969 (45). For the Dutch situation the costs of CF were estimated to account for 0.07% of the health care budget (46). Life time costs for CF patients, assuming the life expectancy to be 25 years have been estimated to be approximately €225,000 (8). In another study, costs of care in patients with CF to the age of 35 were estimated to be €430,211 (47).
Costs of a disease are divided in direct and indirect costs. For CF the clinical visit, hospital admission, outpatient drug costs (for example; rhDNase, antibiotics and dispensing fees) and laboratory tests are seen as direct costs. Direct non-medical costs include out-of-pocket expenses for dietary recommendations and travel costs to receive care. The indirect costs include for example productivity losses (48). These indirect costs were often disregarded in the reviewed publications on CF costs.
Direct costs, such as costs for medication, account for most expenses of medical care in CF patients. Total costs were estimated to be around €5,250 per patient per year (49). Due to interindividual differences not all patients benefit from rhDNase (24, 48-51). The differences in costs depend on changes in respiratory function and possibility of exacerbations. The Dutch costs of rhDNase were estimated to be about 2,5 million Euro's in 1997 and were estimated to remain constant the following years (52).

Next to medication, rates of exacerbations of CF and symptoms of this disease and hospitalization are the largest cost components. More than 1 out of 3 patients with CF are hospitalized each year (16, 53). Over 3 out of 4 patients admitted are treated for pulmonary infections. The costs per admission were estimated to range from €7,840 to €28,000 (16, 53, 54).
Different studies estimated the percentages of different cost-components for direct costs in CF. Table 1 shows these results

Table 1. Direct costs of CF: percentage of distribution

Reference:	(48)	(45)	(47)	(55)
Hospital care	*33%*	-	*42%*	*47%*
RhDNase	*44%*	-	-	*18%*
Clinic visits	*12%*	-	-	*12%*
Laboratory tests	*8%*	-	-	-
Medication (other than rhDNase)	*3%*	*57%*	*37%*	*10%*
Other	-	*43%*	*21%*	*13%*

As can be seen in table 1, medication costs and hospital care are the largest component of the costs. RhDNase offers a therapeutic opportunity to reduce RTI-related health care use in CF patients. The objective behind therapy with rhDNase is improving health related quality of life and additionally lowering the costs of CF. As described above, several cost components (hospitalization, exacerbations, symptoms, therapy, RTI-related, antibiotics, etc) have to be taken into account when estimating the cost-benefit ratio of rhDNase therapy in CF patients. RhDNase contributes significantly to the costs of treatment of CF (56). Therefore, if these costs can be reduced by development of a dry powder formulation for inhalation, significant benefits may be gained.

Quality of Life considerations

The respiratory symptoms of CF can adversely affect daily activities of the patient with CF, because of the unpredictability of these symptoms and serious consequences of exacerbations. It is difficult to predict the degree to which the quality of life of an individual can be affected by CF.
Some studies mention that the great majority of CF patients live well in adulthood with an acceptable quality of life, due to basic therapies. New therapies can help to improve the quality of life (9, 57). However, all

patients suffer from deterioration in their disease, which leads to limitations and thus ultimately reduces quality of life significantly (9).
The majority of morbidity and mortality in CF results from inflammation and infection of the airway lumen, which leads to a certain degree of disability in patients with CF (58). In one study, the impact of pulmonary exacerbations on quality of life in CF patients was studied (59). The objective was to compare health-related quality of life (HRQOL) of CF patients to general population and to determine the relationship between HRQOL and clinical and demographic factors.
For measurement of HRQOL, five aspects of health were included: physical functioning, role functioning, mental health, social functioning and general health perceptions. HRQOL measures the individual ability to function in each area and the individuals evaluation of their functioning (59).
The study of Britt et al. [56], showed that exacerbations do not have a profound negative impact on HRQOL that is not explained by differences in function of lungs, nutrition status or demographic factors. In spite of this, lung function, nutrition, 6-min walk, age, gender, and insurance status were not significantly associated with HRQOL in this study population (59).
On short term, well-being and quality of life of CF patients is improved by rhDNase, through improvement in lung function and a reduction of RTI-incidence (14, 50).
In the study of Oster et al. (16), questionnaires were developed to evaluate well-being (feeling, energy, activity, sleep, appetite) and physical symptoms (sputum production, frequency and severity of cough, chest congestion). The results mentioned that compared to the placebo-group, there was significantly less dyspnoea, significantly improved well-being and there were significantly fewer CF related symptoms in the rhDNase-group. This implies that when CF patients are treated with rhDNase they have a better quality of life compared to patients who are not treated with rhDNase.
Another study noted that baseline HRQOL assessments show that adults with CF, reported decrements in health status and functioning. In this study, a significant association was found between the rhDNase therapy and one-year change in HRQOL. One of their suggestions was that a further evaluation of the relation between hospital admission and changes in HRQOL is required (17).

As opposed to life expectancy, morbidity and mortality, quality of life is mainly not affected by the CF disease as such (e.g. dietary requirements), but much more by the symptoms and respiratory tract inflammations and infections. Hospitalization affects quality of life also in a negative sense. RhDNase has a positive short-term effect on the quality of life as mentioned above. In the reviewed studies, long-term effects and delivery of rhDNase were not evaluated with respect to quality of life in patients with CF.

METHODS

We searched for articles on recombinant human DNase (rhDNase) in international literature databases (Medline and Embase), which were published in English and Dutch in the beginning of the nineties. Additional references were identified from the reference lists of these articles.
The search terms were 'cystic fibrosis', 'recombinant human DNase (rhDNase)', 'dornase alpha', 'pharmaco-economics', 'cost-effectiveness', 'cost-utility', 'health- economics', 'dry powder inhalation', 'aerosol inhalation', 'nebulization' and 'delivery (systems)', or one of the possible combinations of these terms. The searches were last updated February 18th, 2003.
The selection was based on articles with economic analyses in patients with cystic fibrosis that receive rhDNase by inhalation. All pharmacoeconomic analyses, such as cost-effectiveness, cost-utility and cost-benefit analyses, were included. Articles on efficacy, tolerability, safety and compliance with respect to rhDNase, were also included in this review. Papers on efficacy, tolerability, safety and compliance are reviewed in the next section, as these provide the basis of pharmacoeconomic assessments. Section 5 reviews the general pharmacoeconomics, whereas section 6 considers the drug-delivery systems with regard to economics.

CLINICAL PROFILE OF INHALED RHDNASE

Efficacy

The Cystic Fibrosis Foundation has suggested a definition of the disease severity based on the Forced Expiratory Volume in 1 second (FEV_1) and the corresponding Forced Vital Capacity (FVC):

- Mild: $FEV_1 \geq 70\%$ of predicted value or FVC $\geq 85\%$ of predicted

- Moderate: 40% ≤ FEV_1 < 70% of predicted value or 60% ≤ FVC < 85% of predicted
- Severe: FEV_1 < 40% of predicted value or FVC ≤ 60% of predicted (8).

Most of the reviewed studies used a daily dose of 2.5 mg rhDNase in patients with CF and observed the short term effects of rhDNase. Long-term effects (benefits) are not yet available. Consequently, it is uncertain to what extent the short-term effects can be used to predict any long-term benefit (60).

Fuchs et al (54) found a reduction in exacerbations of respiratory symptoms and a slight improvement in pulmonary function after 2.5mg rhDNase therapy in a randomized, double-blind, placebo-controlled study in which 968 patients participated. One or more exacerbations occurred in 27% of the patients who received a placebo, 22% in patients who received once daily rhDNase and 19% in patients who received twice daily rhDNase (2 x 2.5 mg). The FEV_1 improved with about 6% in patients who received rhDNase (24, 53, 54). The study of Menzin et al (6) showed that the reduction of the viscosity of purulent sputum by rhDNase as is studied in early clinical trials of aerosol delivery, accomplishes improvement in pulmonary function (FeV_1) of 10 to 15% in patients with CF. In this study, a reduction in RTIs as consequence of rhDNase therapy was also found.

Cobos et al (61) studied the minor to moderate benefits in short-term clinical trials. In this study, 166 patients continued rhDNase therapy during the data collection. After one and after two years of treatment with rhDNase, the mean changes in FEV_1 were 3,3% and 5,1%, respectively. At the end of the same periods 34% of patients had improved their baseline FeV_1 by 10% or more but in about 50% of the patients the level fell below the baseline. The medium-term response to rhDNase treatment was correlated with an early response during the first 3 months. There was a large inter-individual difference in change in pulmonary function documented and there were no consistent changes in exacerbation pattern found during the first year of rhDNase treatment.

Johnson et al (48) performed an observational study in 283 CF patients (>6 years; FEV_1 < 40% predicted). The treatment group received 18 months rhDNase, while the control-group, consisting of 2382 patients, had never received rhDNase. The FEV_1 for patients treated with rhDNase improved with 3,9% of the predicted value while the untreated control group had a decline in FEV_1 of 1,6% of the predicted value. This study

provided some evidence for effectiveness, without statistic significance, of rhDNase.
Milla (35) studied patients with CF retrospectively on spirometric parameters for two years. 190 Patients were included, who after two years of rhDNase administration showed a positive trend with respect to malnutrition and hardly hospital admissions. There was a mild decline in FEV_1, while FeV_1/FVC remained stable.
In the study of Geller (23) characteristics of two different aerosol delivery systems that might affect efficacy in patients with mild CF, were reviewed. The reviewed systems were a Hudson T Up-draft II with a Pulmo-Aide compressor or the Durable Sidestream nebulizer, powered by a CR50 compressor. The delivery system with the smaller droplet size - the Sidestream nebulizer - tended to provide more improvement than the system with a larger droplet size. The results of the study indicate a statistically not-significant improvement in CF patients who received the smaller particle size aerosol (4.3% versus 2.5%, $p = 0.06$).
Johnson et al (7) showed in a phase III trial that rhDNase therapy (once or twice daily; 24 weeks) in CF patients resulted in significant reductions in the number of respiratory symptoms and exacerbations in lung disease. This resulted in a reduction in the use of parenteral antibiotics and significant improvement in pulmonary function and also a reduction of hospitalizations.
Suri et al (12) compared the consequences of three therapies in children: 1) daily rhDNase (2.5 mg), 2) alternate day rhDNase (2.5 mg) and 3) hypertonic saline (two times per day) during 12 weeks. The study was an open randomised cross-over trial, in which 48 children with CF were allocated. This resulted in a significant greater increase in mean FeV_1 (2-14%) with daily rhDNase compared with hypertonic saline, while daily and alternate daily rhDNase did not differ significantly.
In another clinical trial, Grieve et al (51) investigated the effect of 12 weeks treatment with daily rhDNase, alternate day rhDNase or hypertonic saline (HS) in a randomized, crossover study, in which 40 children with CF were allocated. They found a 14% (5-23%) improvement in FEV_1 for daily rhDNase compared with HS, while alternate day rhDNase compared to HS resulted in a 12% (2-22%) improvement in FeV_1. There was, as also described by Suri et al (12), little difference between FEV_1 of daily and alternate day rhDNase.

The publication of Conway (50) provides evidence that rhDNase is effective in patients older than 5 years of age. Early studies of rhDNase showed an increase in baseline FEV_1 of about 9-14% and that the improvement in pulmonary function maintained at a plateau about 6% higher than placebo for continued rhDNase treatment. A similar effect is seen for FVC. Also it was mentioned that a 24 week phase III study resulted in a decrease of RTIs and exacerbations, which reduced antibiotic therapy by 28 to 37% and also reduced the number of hospitalizations. The longer term effects in 188 patients showed persistent benefit for FVC of 9% of predicted and for FEV_1 of 5% of predicted. In a clinical study of a group of 65 children treated with rhDNase for 6 months an increase of 14% in FeV_1 was shown and in 45 of 59 adults with 15 months treatment, an increase of 15% in FeV_1 was achieved, which was maintained by rhDNase therapy (50).
The Pulmozyme (rhDNase) Early Intervention Trial (PEIT) of Robinson et al (62), showed reduction in the risk of pulmonary exacerbations and thereby a reduction of 34% in parenteral antibiotic therapy. This study also showed an improved forced expiratory flow at 25 to 75% of FVC and improvement in FeV_1 over a 2 year period in CF patients with almost normal lung function. The results of this study, conducted at 49 sites in 12 countries in a randomized trial (placebo-controlled) design, showed benefits from rhDNase in CF patients from early intervention in the course of their lung disease.
In another, this time short term, study of rhDNase of Robinson et al (63), an increase of 7.5% in FeV_1 and 5.4% in FVC from baseline was seen in patients who received rhDNase compared with placebo. The authors were unable to demonstrate improvements in either ciliary or cough clearance with regard to the short term use of rhDNase.
The reports of the efficacy as mentioned above are encouraging, with short-term effects of rhDNase relatively positive as indicated in the reviewed studies. In the mild and moderate state of CF improvement in the pulmonary symptoms (measured in FeV_1, FVC and number of exacerbations) can be of great influence in the reduction of antibiotic therapy and hospitalization of CF patients. This results in a reduction of costs of care in CF. However, the efficacy in severe pulmonary disease is more uncertain, some studies show improvement, some show no differences and some indicate even slight worsening in the number of exacerbations (8). This might be due to large inter-individual differences

with respect to the efficacy of rhDNase. The limited amount of information about the long term effects is a problem in predicting or defining the efficacy of rhDNase.

Tolerability, Safety and Compliance

Adverse drug events during rhDNase therapy are generally mild and the drug is well tolerated. The most common adverse effects are respiratory related (24). In infants and young children, exposure to rhDNase appeared to be safe over a 2 week period (56).
RhDNase is currently only administered by nebuliser (aerosol). Therefore, it is difficult to find out whether adverse events were related to the drug or to the method of administration. The severity of the disease is of importance in this possible relation. The variability in adherence to rhDNase treatment is a debatable subject in the validation of the safety and efficacy of rhDNase. Nebulization as an administration technique leads to lower compliance to the therapy of CF patients, whilst the compliance is of utmost importance, since the efficacy of the treatment heavily depends on the compliance (64).
Post-marketing clinical experiences have confirmed the relative safety of rhDNase that was documented in clinical trials. Voice alteration, hoarseness, laryngitis, pharyngitis and rash are the most common adverse events in patients using rhDNase (8, 56, 57, 62). These symptoms are self-limited and do not require medication. Next to these adverse events, chest pain and conjunctivitis also have been reported. These events were not more frequently reported than with placebo. The percentage of withdrawal from therapy in the clinical trials was not significantly different for rhDNase compared to placebo (8).
Information on adverse events of rhDNase after long term therapy is scarcely documented. In a two year study in patients with mild to moderate CF, no serious adverse drug events in treatment with rhDNase were found (34).
Up to now, rhDNase has shown to be a well-tolerated drug, which can be used safely with few adverse drug events that do not require new drug therapies to treat those adverse events.

PHARMACO-ECONOMIC ANALYSIS OF AEROSOL RHDNASE

The few cost(-effectiveness) studies available concerning rhDNase, are often published as abstracts or short reports while fully-reported cost-

effectiveness analyses are lacking. For that reason only little information is available about the methodology and about cost categories. In short, some costing has been done with respect to aerosol rhDNase but a full cost-effectiveness analysis is (yet) lacking (6, 7, 12, 14, 16, 45, 51, 53, 55). Below, the empirical evidence on pharmaco-economics of rhDNase is taken from the four most comprehensive studies on the topic (12, 14, 16, 51). These studies are evaluated with respect to several criteria that we feel are currently the most important for conducting "good" pharmaco-economic research: for example, the societal perspective should be taken (including all relevant cost categories, even indirect costs of production losses if important), taking the adequate time frame (including long-term effects) and results should preferably be expressed per life-year gained (LYG) or per quality-adjusted life-year (QALY) gained (65).

According to Oster et al (16), rhDNase reduced the estimated average costs of RTI-related care over 24 weeks by €912 – €1,884 compared with placebo. Limited to only protocol-defined RTIs, the reduction of the costs was estimated at €681 - €1,057. The findings in this study suggest that rhDNase therapy may prevent approximately 31-33 RTI-related hospitalizations per 100 patients on an annual basis. This would correspond with a saving of €2,016 - €4,144 per patient annually. Also, this paper mentions that the reduced costs of RTI-related care are expected to be about 33% (18.3-37.5%) of the total costs of rhDNase-therapy. As mentioned, this analysis does not include all relevant cost categories that may be specified.

McIntyre (14) used the results of the study of Oster et al (16) to estimate the net costs per LYG as a cost-effectiveness outcome. For that purpose annual costs of rhDNase of €5,040 were assumed. The discounted lifetime costs for a CF patient was estimated at €163,100, including the acquisition costs of rhDNase and the additional costs of treatment for 3 extra years of life. The net costs per LYG were subsequently estimated at €19,110 and the additional cost of rhDNase treatment at €1,736 per year. The authors conclude that taking into account the potential long-term benefits, rhDNase may be conceived a cost-effective option.

Suri et al (12) compared mean incremental costs of daily and alternate day rhDNase with hypertonic saline (HS) over a 12 week period as investigated in a clinical trial(66), in what they labelled themselves a cost-consequence analysis. The mean incremental costs of daily rhDNase compared with HS were €986 and the incremental cost of using daily over

alternate day rhDNase was €359. A formal cost-effectiveness analysis to analyse whether the increased effectiveness of daily rhDNase compared to HS twice daily delivered by nebulizer was not done. It was merely concluded that daily rhDNase was more expensive and significantly increases health care costs (12). Administering rhDNase on an alternate day rather than on daily basis may be as effective, with a potential cost saving.
In the study of Grieve et al (51) daily rhDNase was found to be more effective than HS and almost as effective as alternate day rhDNase. For a ceiling ratio of €140 per 1% gain in FeV_1, the mean net benefits of daily and alternate daily rhDNase compared with HS were €811 and €832, respectively. The mean net benefit of alternate compared with daily rhDNase was €21. It was concluded that if decision makers were prepared to pay €140 per 1% gain in FeV_1 over a 12 week period (ceiling ratio), both rhDNase therapies may be conceived cost-effective.
Generally, the long-term perspective is often missing in these studies and they often only include the cost savings by reducing exacerbations and RTI's. Ergo, hospital admissions and reduced costs for antibiotic therapy were included, but direct costs for, for example, adverse drug reactions were not. Additionally, direct non-medical costs such as travelling to the health-care service, and indirect costs of productivity losses were not estimated. Finally, we note that only one study estimated net costs per LYG, and none estimated QALYs gained, despite the fact that important health-related quality-of-life effects exist.

ECONOMIC ASPECTS OF RHDNASE DELIVERY SYSTEMS

The cost-effectiveness analysis done as mentioned above were all based on rhDNase administration via a nebulizer. As mentioned previously, the Dry Powder Inhalation (DPI) of rhDNase may offer advantages, in particular more efficient administration (table 2). Focussing at drug-delivery systems for patients with CF (for example, DPI vs. aerosol inhaler) no cost-effectiveness analysis was found. Potential advantages of a DPI of rhDNase are:

- An improved compliance to therapy,
- Improved (deep) lung deposition,
- No need for electricity or compressed air,
- A reduced administration time
- No need to cool the rhDNase-ampoule (29, 36, 56, 67, 68)

On the other hand, a short-coming of DPI's is that the patient's own inhalation strength is needed for the deposition of rhDNase in the lungs. This can be a problem for patients with severe CF-related symptoms and exacerbations.

Table 2. Advantages and disadvantages of DPI vs nebulization, partly taken from De Boer et al. (69)

Dry Powder Inhalation		Nebulization	
Advantages	Disadvantages	Advantages	Disadvantages
Less expensive	Performance depends on the patient's inspi-ratory flow profile	High dose uniformity	Expensive
Propellant free	Resistance of the device and other design parameters	Performance does not depend on the patient's inspiratory flow profile	Low deposition efficiency
High deposition efficiency	Potential difficulties to obtain dose uniformity		Long administration time
Short administration time (mobility)	Not worldwide available		Need for power supply or pressurized air (immobility)
Less potential problems with drug stability			Potential problems with drug stability
Less potential for extractables from device components			Large number of combinations of devices possible

To appreciate the economic aspects of delivery systems in general we note the study of Massie et al (70) comparing a nebulizer (aerosol delivery) with DPI in asthma. In this study it is mentioned that the equipment for aerosol delivery is costly (air compressor and nebulizer), less than 5% of the nebulized dose is actually delivered to the airway (relatively high wastage) and the duration of delivery confronts the patient with problems. These problems can negatively affect patients' adherence to the treatment. In short, the nebulization delivery may be an inefficient and relatively expensive delivery system, if DPI is available (70).
DPIs are self-actuating, provided that sufficient inspiratory flow is generated. DPIs are less expensive than nebulizers and they offer advantages in terms of better deposition, better disease control and the ease of use. The low cost of DPIs may be further decreased by a reduction in the number of costly complications and exacerbations, due to better compliance. Furthermore, DPIs enhance patient comfort. A drawback of DPI is the requirement for the ability to develop sufficient inspiratory flow, which is often impossible for children under the age of 6-8 years (70).

EXPERT OPINION

CF is a costly disease, in which rhDNase is one of the therapeutic options. But the wastage of rhDNase and other disadvantages of nebulization make the therapy expensive. A better, efficient and easier delivery system for rhDNase can possibly improve efficiency and reduce wastage of rhDNase. For that reason, it could be more cost-effective than nebulization. From an ethical point of view it is better to provide rhDNase by DPI, since the adherence of patients to nebulization is quite low as described by Burrows (64). Low adherence results in in-effective therapy and could lead to increased costs. This can be one of the reasons why DPI could be more cost-saving than nebulized rhDNase. Other advantages of DPI like the absence of the need for electricity, easier usage and more efficient delivery of rhDNase (compared to the nebulization) are also relevant with respect to potential cost-savings of rhDNase therapy.
For drugs which have a significant impact on the total health care budget, large efforts should be made to develop an adequate dosage form. In this way, innovative research is stimulated which potentially results in lower cost as can be proved with rhDNAse. However, the focus when introducing

a new drug is primarily directed toward the regulatory agencies and their requirements regarding efficacy and safety, which is completely understandable for reasons of public protection. On the other hand, the horizon should be put beyond the regulatory hurdle and put to the group of patients who are to receive the new drug. If beforehand it is already known that compliance is a problem with a certain dosage form, action should be taken to avoid that problem. At the time of introduction of rhDNase to the market, the technology to create a dry powder of labile substances was not fully available. However, this technology has improved over the past decade and is readily available. Moreover, DPI technology has improved meanwhile. Therefore, DPI is now an option when such drugs are developed. This gives rise to great opportunities, as for example shown by insulin (71-74).
Our opinion is that DPI will gain a greater share in the field of inhalation due to obvious reasons like patient adherence, ease of use, efficiency and cost-effectiveness. As mentioned in chapter 2, the deposition efficiency of DPI may be 4- to 6-fold that of a nebulizer (28, 35, 36). For illustrative purposes we crudely estimated cost-effectiveness outcome of once-daily DPI rhDNase administration based on McIntyre (14), in which net costs per LYG for once-daily aerosol delivery were estimated at € 19,110 in the baseline. Due to higher deposition efficiency of DPI, we conservatively assumed that for DPI delivery 30% less rhDNase is necessary to reach at least the same benefits and effectiveness as in delivery by nebulizer(14, 16). Then, for DPI rhDNase therapy net costs per LYG may be estimated at € 12,100. This corresponds with a reduction in the cost-effectiveness ratio of almost 40% for DPI delivery of rhDNase compared to delivery by means of nebulization. Incremental cost-effectiveness for DPI over nebulization may then be estmated at € 5,100.

FIVE-YEAR VIEW

With respect to the cost-effectiveness analysis in rhDNase (aerosol-delivery) treatment, gaps were detected in the methods and cost components included. For the future it is advisable to carry out a cost-effectiveness analysis in which al cost components (direct and indirect) are included and the outcome should preferably be expressed in costs per Quality Adjusted Live Year (QALY) or costs per life-year gained. Extrapolating from short-term results in clinical trials to long-term pharmaco-economic outcomes, for example, life-years gained, requires

modelling within an adequately chosen time frame. Such a model should include up to date epidemiological data, for example, from CF-registries that exist for several countries, such as the Netherlands.
It may be estimated that DPI will result in better adherence to the expensive therapy. The higher deposition efficiency of DPI compared to nebulization will result in a cost-reduction. Furthermore, to nebulize a drug there are numerous options to choose from considering only the numerous available nebulizing techniques and nebulizers. Moreover, these different nebulizers have different performances with respect to the rate of nebulization and the characteristics of the aerosol they create. For the physician it is difficult to choose an appropriate nebulizer which gives an appropriate particle size distribution. In contrast, a DPI is developed alongside with the formulation. This means that the inhalator is specifically designed to give the best result in terms of de-agglomeration of the formulation. The inhalator is so to say "dedicated" to the specific formulation. For the physician it has advantages and the patient eventually benefits most of DPI. It is expected that DPI-use will increase over the next period and that nebulization will still be a therapeutic option for a small number of drugs. For high-cost medication, such as rhDNase, it will be more likely to be developed as a DPI, as the cost-reduction may be expected to have the greatest impact compared to nebulization for these drugs.

Key Issues

Patients with Cystic Fibrosis have thick, tenacious sputum. They are chronically colonized with bacteria. This leads to frequent exacerbations and is a burden for these patients. Of the patients which are hospitalized each year (around 33%), 75% is treated for pulmonary infections.
Recombinant human Deoxyribonuclease I (rhDNase) is a protein which has beneficial effects on lung function (FEV_1/FVC) and number of hospitalizations.
RhDNase is currently administered by nebulization which is an inefficient method of administration. Dry powder inhalation of rhDNase is almost available and is likely to be more effective than nebulization.
In this review we investigated the pharmacoeconomic effect of dry powder inhalation versus nebulization of rhDNase.
We performed an extensive review of the available literature but scarce information about pharmacoeconomics of rhDNase is available. It was

found that rhDNase does have a moderate effect and that dry powder inhalation can possibly be cost-saving with the same efficacy as obtained by nebulization.
We conservatively assumed that for DPI delivery 30% less rhDNase is necessary to reach at least the same benefits and effectiveness as in delivery by nebulizer. This corresponds with a reduction in the cost-effectiveness ratio of almost 40% for DPI delivery of rhDNase compared nebulization.
We hypothesize that the patients benefit most from dry powder inhalation due to increased mobility and ease of use. It is expected that DPI-use will increase over the next period and that nebulization will still be a therapeutic option for a small number of drugs. For high-cost medication, like rhDNase, it will be more likely to be developed as a DPI, since there might be a cost-reduction compared to nebulization.

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CHAPTER 7

Dry powder inhalation of hemin to induce heme oxygenase expression in the lung

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ABSTRACT

The purpose of this study was to formulate hemin as a powder for inhalation and to show proof of concept of heme oxygenase 1 (HO-1) expression in the lungs of mice by inhalation of hemin. Hemin was spray dried from a neutralized sodium hydroxide solution. The particle size distribution of the powder was between 1-5 µm. Dispersion from the Twincer dry powder inhaler showed a fine particle fraction (<5 µm) of 36%. A specially designed aerosol box based on the Twincer®-inhaler was used for a proof of concept study of HO-1 induction by inhalation of hemin in mice. The aerosol in the exposure chamber of the aerosol box remained aerosolized up to 5 minutes. A rhodamin B containing aerosol was used to show that the aerosol box gave deposition over the entire lung indicating the suitability of the model. Additionally, inhalation of hemin showed a dose dependent increase in HO-1 protein expression in the lungs. In conclusion, hemin was successfully formulated as a powder for inhalation and the inhalation model allowed controlled HO-1 expression in the lungs of mice. Future studies investigating the utility of inhaled hemin in treating disease states are warranted.

INTRODUCTION

The heme oxygenase (HO) metabolic pathway has recently received an explosion of research interest due to its potent physiological and cytoprotective properties, e.g. (1-3). The inducible (HO-1) and the constitutively expressed (HO-2 and HO-3) forms of HO catalyze the oxidation of heme to biliverdin, carbon monoxide and iron (4). Subsequently, biliverdin is rapidly converted to bilirubin which is a potent endogenous antioxidant (5). All three downstream products (biliverdin/bilirubin, CO and Fe/ferritin) participate in the cellular defense. HO-1 has an integral role in the response to oxidative stress and inflammation and is induced by numerous oxidative and inflammatory stimuli (6).

Recently, it was shown that HO-1 expression in lung tissue and macrophages obtained by bronchoalveolar lavage of patients with chronic obstructive pulmonary disease (COPD) is decreased compared with healthy controls (7, 8). COPD is a respiratory disease characterized by a chronic inflammatory response in the lungs, which is primarily caused by the toxic effects of smoking. It is tempting to hypothesize that in COPD patients an imbalance between systemic reactive oxygen species and relative antioxidant systems exists, which is possibly caused by insufficient upregulation of HO-1 (9). One of the substances that induces HO-1 is hemin (ferriprotoporphyrin IX), the oxidized form of heme (10, 11). Induction of the heme oxygenase pathway by inhalation of natural substrates, such as hemin, could therefore be of great benefit in diseases like COPD (12, 13). So far, inhalation of hemin in humans has been performed only once as a control to show that the HO-1 pathway is involved in asthma (13). In that study, nebulization of 2 ml of 10^{-4} M hemin resulted in increased levels of exhaled CO, indicating that the HO-1 pathway can be induced by inhalation of hemin. However, no direct evidence of HO-1 induction was given.

In the clinical setting, nebulization is characterized by a low deposition efficiency (typically 2-27%), and needs electricity or pressurized air for the aerosol to be created. In contrast, dry powder inhalation is characterized by higher deposition efficiency (up to 60%) and the patient delivers the driving force for the aerosol to be created (14-17). Moreover, nebulization is accompanied by patient immobility, whereas dry powder inhalers are easy to carry and use. Furthermore, dry powder inhalation is likely to result in increased patient compliance, especially in chronic use.

The aim of this study was to formulate hemin as a powder for inhalation and to show a proof of concept for induction of heme oxygenase 1 expression in the lungs of mice by inhalation of hemin. To obtain the proof of concept we administered hemin as a powder for inhalation to mice by using a specially designed aerosol box, which is based on the Twincer® dry powder inhaler (18).

MATERIALS AND METHODS

Materials

Hemin (51280) and rhodamin B (83689) were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). Inulin, with a degree of polymerization of 23, was a generous gift of Sensus (Breda, The Netherlands). Sodium hydroxide (NaOH), hydrochloric acid (HCl) and ammonia (NH_4) were of analytical grade and purchased from commercial suppliers. Demineralized water was used throughout the experiments.

Methods

Quantitative determination of hemin

Sample concentrations for determination of solubility and drug load were spectrophotometrically analyzed in phosphate buffered saline (PBS) with the Ultraspec 4052TDS apparatus at 385 nm (LKB, Zoetermeer, the Netherlands).

Solubility

In order to measure the maximum solubility in different alkaline solutions, 7 solutions with a pH between 11.0 and 14.0 were prepared in steps of 0.5 pH units. To these solutions excess hemin was added under constant stirring. After 30 minutes, the solutions were centrifuged at 10,000 rpm for 10 minutes. The supernatant was pipetted and diluted with a phosphate buffered solution and measured spectrophotometrically.

Powder production by spray drying

Due to an excess of sodium hydroxide in the solution to increase dissolution of hemin, the pH of that solution remains high. If such a solution is spray dried, the resultant powder causes an alkaline pH shift upon reconstitution. To avoid alkaline pH changes upon reconstitution of the spray dried powder, the pH needs adjustments during or before spray drying. Two methods were used for pH adjustment. In the first method a

sodium hydroxide solution (0.7% w/v) with pH 13.5 was immediately before spray drying adjusted to pH 7.6 with hydrochloric acid. The second method consisted of spray drying an ammonia solution (1.2% w/v) with a pH of 13.5. During spray drying, ammonia evaporates and is not present in the dried powder any longer.
Four powders were spray dried. The first powder was prepared by dissolving hemin in a sodium hydroxide solution (0.7% w/v) at a pH of 13.5 to obtain a total concentration of 4.5% w/v. After pH adjustment the solution was filtered through a 1.2 µm filter. Hemin was not removed by filtration from the solution. After spray drying, this powder thus contained sodium chloride and hemin. The hemin drug load was determined to be 63% w/w.
The second powder was produced by dissolving hemin in an ammonia solution (1.2% w/v) with a pH of 13.5. After spray drying, this powder thus only contained pure hemin. This powder was used as a control in the dissolution experiments.
Finally, spray dried controls containing rhodamin B or inulin were prepared by dissolving the respective controls in a sodium hydroxide solution followed by pH adjustment before spray drying. Rhodamin B served as a control for the *in vivo* deposition characteristics and thus the suitability of the aerosol box; inulin served as a control for the local effect of powder inhalation with sodium chloride.
Spray drying was performed with a Büchi 190 Mini Spray Dryer (Büchi, Flawil, Switzerland) equipped with a two-fluid nozzle (0.5 mm). The solutions were sprayed at a fluid flow rate of 3.2 ml/min and an atomizing air flow rate of 800 l/h. The settings of the aspirator (14) and heater (14) resulted in temperatures at the inlet and outlet of 180 °C and 120 °C, respectively.
The obtained powders were stored in a vacuum desiccator at room temperature.

Dissolution
Dissolution experiments were carried out using an USP dissolution apparatus I (Rowa Techniek B.V., Leiderdorp, The Netherlands) at 37 °C and 100 rpm in 1000 mL phosphate buffered solution with 10 mg hemin, corrected for drug load. Samples from the dissolution vessel were spectrophotometrically analyzed at 385 nm.

Laser diffraction, scanning electron microscopy and cascade impactor analysis

Particle size distributions of the spray dried samples were measured with a Helos Compact model KA laser diffraction apparatus (100 mm lens, Fraunhofer theory) equipped with a RODOS dispersing system (Sympatec GmbH, Clausthal-Zellerfeld, Germany) operated at a dispersion pressure of 5 bar. All measurements were performed in triplicate. The particle size distribution in the exposure chamber was measured by placing the complete aerosol box in the laser beam. To avoid scattering by the Perspex walls, the light was directed through two opposing openings.

Scanning electron micrographs (SEM) were recorded with a JEOL JSM 6301-F Microscope (JEOL, Japan). The powder was dispersed on top of double-sided sticky carbon tape on metal disks and coated with 150 nm of gold/palladium in a Balzers 120B sputtering device (Balzers UNION, Liechtenstein).

The aerodynamic particle size distribution was measured with cascade impactor analysis using a multi-stage liquid impinger (Erweka, Heusenstamm, Germany) with a Twincer®-inhaler (18) attached to the induction port. A solenoid valve was used in combination with a timer to control the flow (corresponding with 4 kPa pressure difference) through the inhaler and the cascade impactor for the duration of 3 seconds. The stages of the cascade impactor were filled with 20 mL phosphate buffered saline (PBS). A total amount of ~100 mg was delivered in 10 separate doses. The concentration of the drug on the different stages was measured by UV absorbance at 385 nm; deposition was subsequently calculated as the percentage of the metered dose. The fine particle fraction (< 5 µm at the flow rate corresponding with 4 kPa across the Twincer, FPF) was calculated by interpolation of the cumulative mass plot versus effective cut-off diameter. The FPF is expressed as a percentage of the metered dose. All cascade impactor experiments were carried out in triplicate.

Aerosol box for passive pulmonary delivery of powders for inhalation

As a proof of concept of HO-1 induction by inhalation of hemin a new device was developed to allow passive, pulmonary dry powder delivery to small laboratory animals. The device, hereafter called aerosol box (figure 1), is actually an accessory for the Twincer®, an effective dry powder inhaler which delivers high dosed formulations in highly constant particle

size distributions (fine particle fraction of 40-50%) independent of the pressure drop between 1 and 4 kPa (18). The efficient aerosol generation at low pressure drops makes the Twincer® most suitable for the aerosol box where the operator manually generates the air flow that disperses the powder. The device consists of the Twincer®, a cylinder with piston to provide a pressure drop across the inhaler and transfer of the aerosol to last part of the device, the exposure chamber in which a fan is built. Mice are attached to the exposure chamber. In total, up to twelve mice can be attached to the exposure chamber.
About 15 mg of powder, equivalent to 9.45 mg active ingredient (hemin, inulin or rhodamin B) and 5.55 mg sodium chloride, was dispersed in the aerosol box. In this study, one dose is defined as 5 minutes exposure to an aerosol that has been generated from 15 mg of drug formulation.

Animals
Female A/J mice (aged 8-10 weeks) were obtained from Harlan (Zeist, the Netherlands) and were held at the Central Animal Facility of the Groningen University. The experiments were approved by the Committee on Animal Experimentation of the University Medical Center Groningen and were performed under strict governmental law, adhering to the Principles of Laboratory Animal Care.

Experimental setup
The mice used in this study were divided into 6 groups. Group 1 consisted of one mouse that was not exposed to any aerosol and served as a control. Groups 2, 3, 4 (n=2 for each group) were exposed to 2, 4 and 6 doses of spray dried hemin, respectively. Group 5 (n=2) was exposed to 6 doses of spray dried inulin and served as control for sodium chloride that was present in the spray dried powders. Inulin, a naturally occurring oligosaccharide was chosen because oligosaccharides contribute marginally to the osmotic pressure due to their high molecular weight. Group 6 (n=2) was exposed to 6 doses of spray dried rhodamin. Group 6 was used to investigate the deposition of dispersed powder in the lungs of mice. Deposition of rhodamin was visualized in 4 µm sections of frozen lung tissue by fluorescent microscopy.

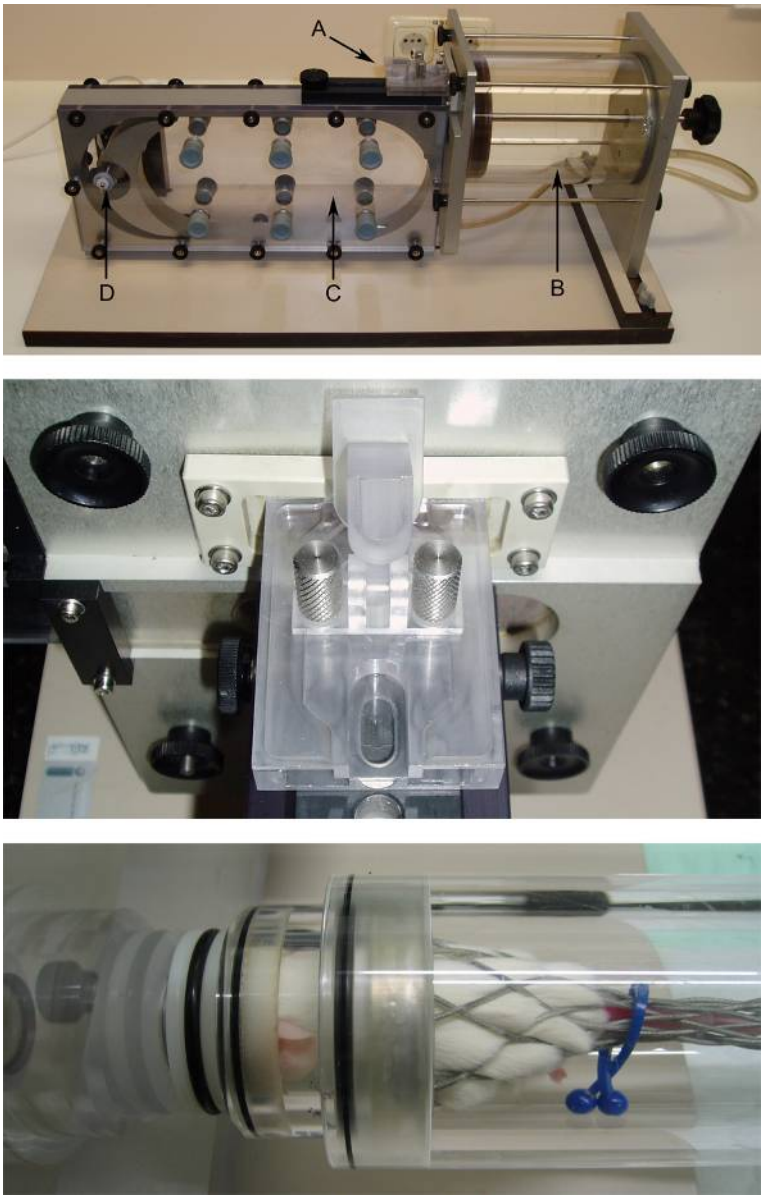

Figure 1. (Top) The aerosol box, a utility for the Twincer® (A). By pulling the piston in the cylinder (B) an aerosol is created and can be transferred to the exposure chamber (C) to which 12 small laboratory animals can be fitted. A fan (D) creates a laminar airflow in the exposure chamber to minimize sedimentation effects. **(Middle)** Detail of the Twincer® attached to the aerosol box. **(Bottom)** Detail of mouse attached to exposure chamber.

Western Blot

Protein samples were made from whole lung tissue using a sonicator (Bandelin sonopuls HD2070, Berlin, Germany). The proteins were separated on molecular weight using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and blotted on a nitrocellulose membrane. The membrane was blocked overnight in 5% low fat milk and the proteins were detected with anti-heme oxygenase-1 (Stressgen, Victoria, Canada) followed by a peroxidase labeled goat-anti-rabbit antibody (DakoCytomation, Heverlee, Belgium). As a protein loading control the membrane was stripped using an 25mM Glycine-HCl buffer containing 1% SDS (pH:2) and stained for ß-actin (Abcam loading control, Cambridge, UK) followed by a peroxidase labeled goat-anti-rabbit antibody (Dako). The bands of interest were visualized using enhanced chemiluminescence.

Band-sizes of each group were quantified using Matlab 6.5.1 (The MathWorks Inc, Natick, USA) and the Dipimage toolbox (Quantitative Imaging Group, Faculty of Applies Sciences, Delft University of Technology, the Netherlands) (19, 20). In short, the grey-scale images were made binary with the Isodata threshold algorithm which calculates the best threshold value out of the grey value histogram of the image (21). Data are given as the ratio between the number of pixels of the HO-1 band of interest divided by the number of pixels of the corresponding ß-actin band.

Histology

HO-1 protein expression was demonstrated in 3 µm sections of paraffin embedded lung tissue with antibodies against HO-1 (Stressgen 896, San Diego, CA, United States). Presence of leukocytes was demonstrated in 4 µm sections of frozen lung tissue with monoclonal antibodies anti-CD45 (Pharmingen).

RESULTS

Formulation. A series of experiments was carried out to measure the solubility of hemin at different pH levels. Hemin is practically insoluble in water (at pH ~7) and organic solvents (22). However, under alkaline conditions hemin is soluble. In alkaline solutions, hemin (FePP-Cl) reacts with excess hydroxide to form hematin (FePP-OH). Hematin acts as an

intermediate in a polymerization reaction which results in a oxy-bridged dimer and probably other polymers (22).

$FePP\text{-}Cl + OH^- \rightarrow FePP\text{-}OH + Cl^-$
$2FePP\text{-}OH \rightarrow (FePP)_2O + H_2O$

The solubility of hemin (figure 2; solid line, primary y-axis) reached a maximum at pH 13.5. At pH>13.5 a large variation in solubility was observed which indicates the occurrence of degradation reactions, such as hydrolysis, at high pH values.

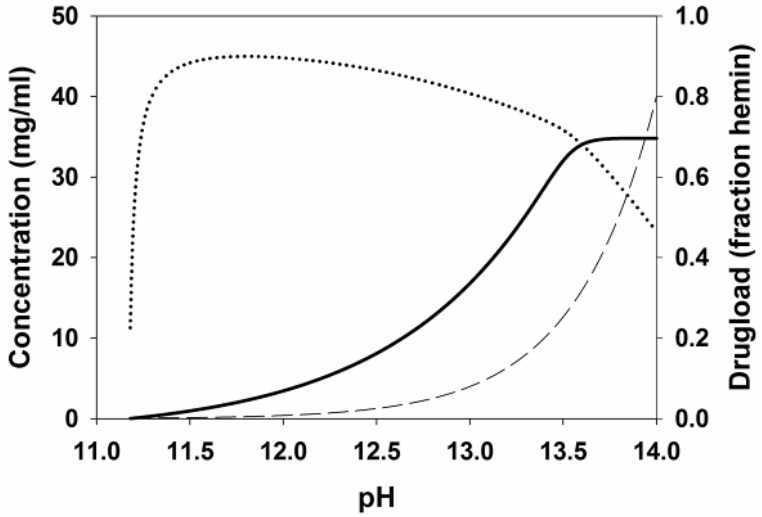

Figure 2. Solubility of hemin (solid line, primary y-axis) as a function of pH and calculated content of hemin in spray dried powder (dotted line, secondary y-axis) as a function of pH. The calculated concentration of NaOH versus pH is represented by the dashed line (primary y-axis).(n=3)

Since the pH of the sodium hydroxide solution determines the amount of hemin that can be dissolved, the sodium hydroxide concentration is the main parameter that determines the efficiency of the spray drying process. The higher the sodium hydroxide concentration, the higher the hemin concentration in the solution. On the other hand, it should be realized that an increase in the sodium hydroxide concentration requires increased amounts of hydrochloric acid for neutralization. This effect will lead to a shift towards higher sodium chloride fractions in the powder,

which of course reduces process efficiency in terms of drug load. Finding the most efficient process condition is a matter of balancing between the solubility of hemin, which is positively affected by the sodium hydroxide concentration, and the hemin drug load in the powder, which is negatively affected by the sodium hydroxide concentration. These effects are shown in figure 2 (dotted line, secondary y-axis).

With increasing pH the drug load of hemin in the spray dried product initially rose up to a maximum of 90% at a pH of 11.8. At this pH the total concentration of hemin and NaOH was only 2.5 mg/ml. Spray drying of a solution with such a low concentration does not result in a high yield. Therefore the pH was increased to 13.5, which resulted in a solution with a total concentration of 45 mg/ml and a drug load of 63% after spray drying as was confirmed by chemical analysis. The spray dried hemin powder consisted of highly irregular particles (figure 3), which were in a size range that corresponded with laser diffraction (table 1).

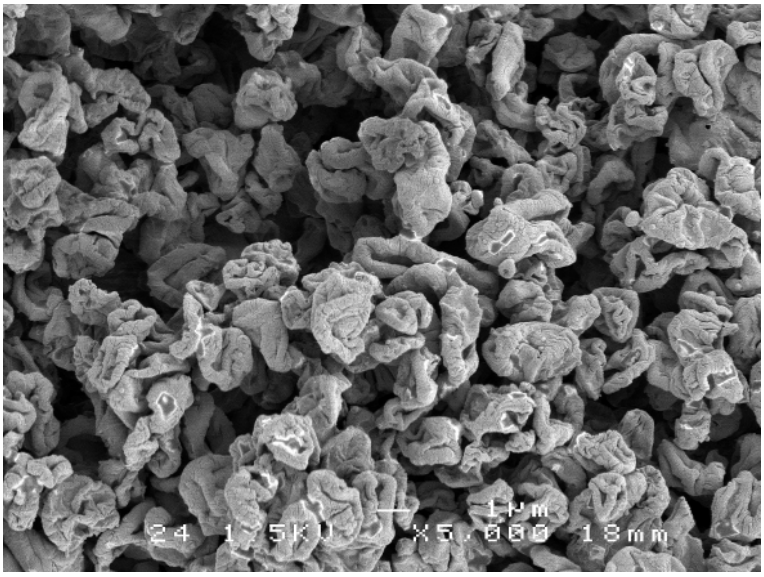

Figure 3. Scanning electron micrograph of spray dried hemin at a magnification of 5000x.

Table 1. particle size obtained from RODOS dispersion (5 bar) at 10, 50 and 90% cumulative undersize of the spray dried powders. All powders were spray dried from a sodium chloride solution, except pure spray dried hemin, which was spray dried from ammonia.

Formulation	x_{10} (µm)	x_{50} (µm)	x_{90} (µm)	Span*
Spray dried hemin	1.01	2.14	3.67	1.25
Spray dried hemin from ammonia	0.95	2.06	3.50	1.24
Spray dried inulin	0.92	2.29	4.66	1.64
Spray dried rhodamin B	0.87	2.02	3.42	1.26

* Span = $(x_{90}-x_{10})/x_{50}$

Spray dried hemin from a neutralized sodium chloride solution displayed a faster dissolution profile compared to spray dried hemin from NH_3 as shown in figure 4. After 2 minutes 50% of spray dried hemin was dissolved, compared to 20 minutes for spray dried hemin from NH_3, while the powders had approximately the same particle size (Table 1).

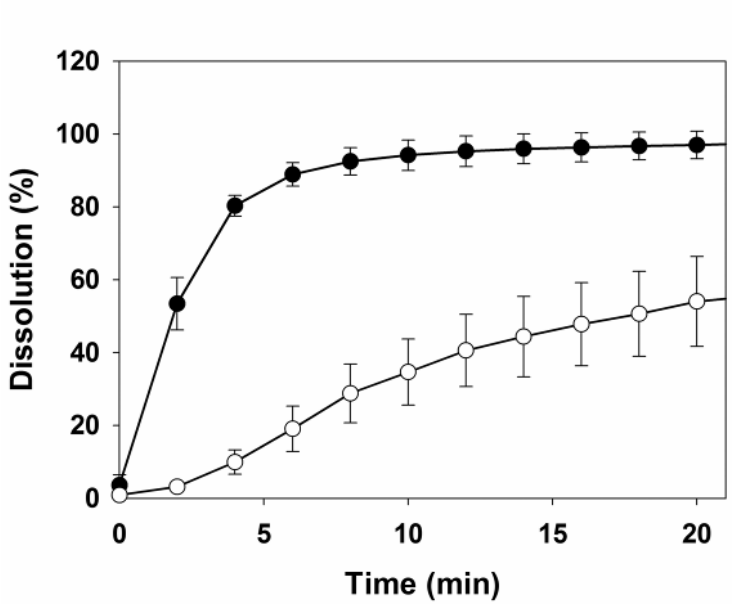

Figure 4. Dissolution rate of spray dried hemin in the presence of NaCl (●) and pure spray dried hemin from ammonia (○). (n=3)

Complete dissolution of the powder spray dried from the NH_3 solution took 110 minutes. The powder with the fastest dissolution rate was used to perform further experiments (aerosol behavior and proof of concept).
In table 1 the particle size distributions of the spray dried powders are shown. The particle size distributions are comparable, only spray dried inulin had a somewhat wider size distribution.
Cascade impactor analysis (figure 5) showed a delivered dose from the Twincer at 4 kPa of approximately 77% with a fine particle fraction (<5 µm) of 36% of the metered dose.

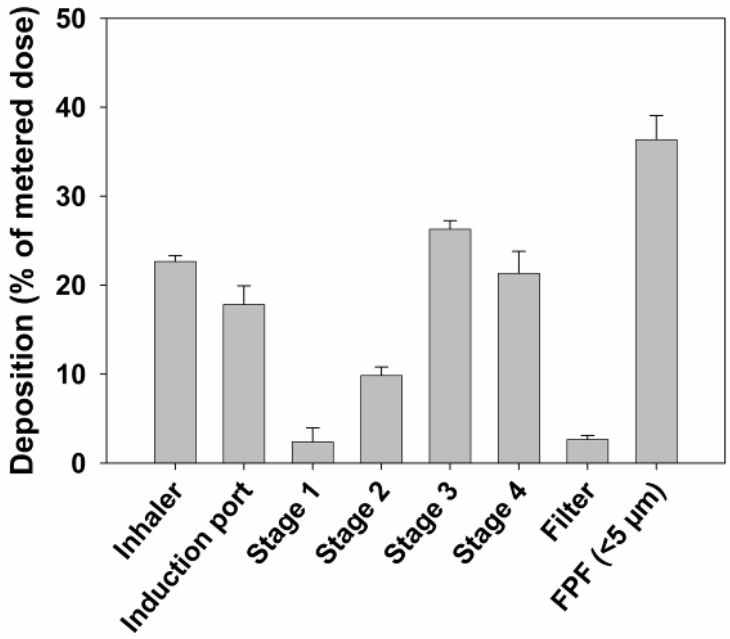

Figure 5. Cascade impactor analysis of spray dried hemin with NaCl after dispersion by the Twincer® at 4 kPa. Fine particle fraction (FPF) represents particles <6.8 µm. Error bars represent highest and lowest value.

Validation of aerosol box.
After dispersion in the aerosol box (figure 6B), most particles (<90%) were between 1 and 5 µm. In the aerosol box the size distribution moved towards finer particles in time, indicating sedimentation. Larger agglomerates (5-10 µm) sediment fast and such agglomerates are no longer present after 1 minute. The change in particle size distribution will not substantially affect the inhalable fraction in the inhaled air however,

since the volume fraction of such agglomerates in the aerosol is small. Sedimentation was confirmed by a decline in the optical concentration (i.e. amount of laser light that is blocked by the aerosol). After 5 minutes the optical concentration was 0.0%, whereas the starting concentration was 1.54% on average. The amount of aerosol in the exposure chamber after 5 minutes was therefore negligible due to sedimentation. Therefore, we exposed the mice for 5 minutes per dose to the aerosol because a longer period would have no additional effect.

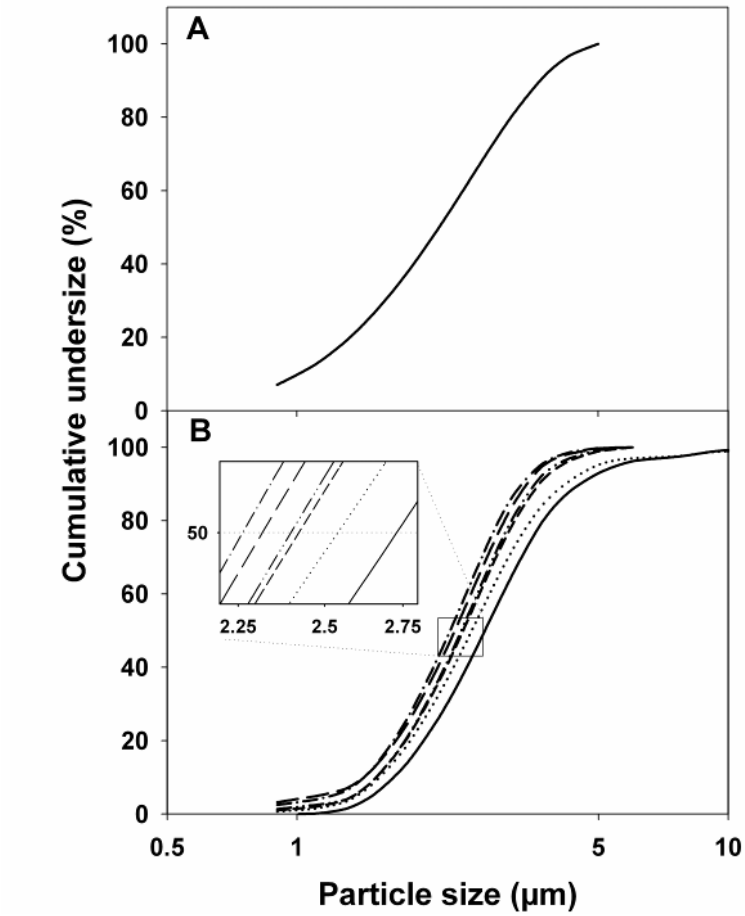

Figure 6. (A) Geometrical particle size distributions of spray dried hemin as measured by RODOS dispersion at 5 bar and (**B**) in the exposure chamber of the aerosol box immediately (———), 1 minute (·····), 2 minutes (— — —), 3 minutes (— ·· —), 4 minutes (—— ——) and 5 minutes (— · — ·) after dispersion by the Twincer®. (Fig.4A: n=3; Fig.4B n=4)

To obtain an indication of the amount of drug that a mouse actually inhales, an *in vitro* measurement was performed by withdrawing 20 ml/min of air during 5 minutes from the exposure chamber. It was shown that this volume contained about 0.02% of each dose, an amount that consequently can be assumed as the administered dose. The assumption of 20 ml/min was on the low side, since respiratory minute volumes of mice of 45 – 50 ml/min have been described (23, 24).
In vivo deposition of inhaled powder in the lungs from the aerosol box was demonstrated by exposing mice to rhodamin B. Rhodamin B had a similar particle size distribution as spray dried hemin (Table 1). By using fluorescent microscopy, rhodamin deposition on the epithelial lining of the airways was demonstrated (figure 7). Rhodamin deposition was detected from the trachea until the large airways.

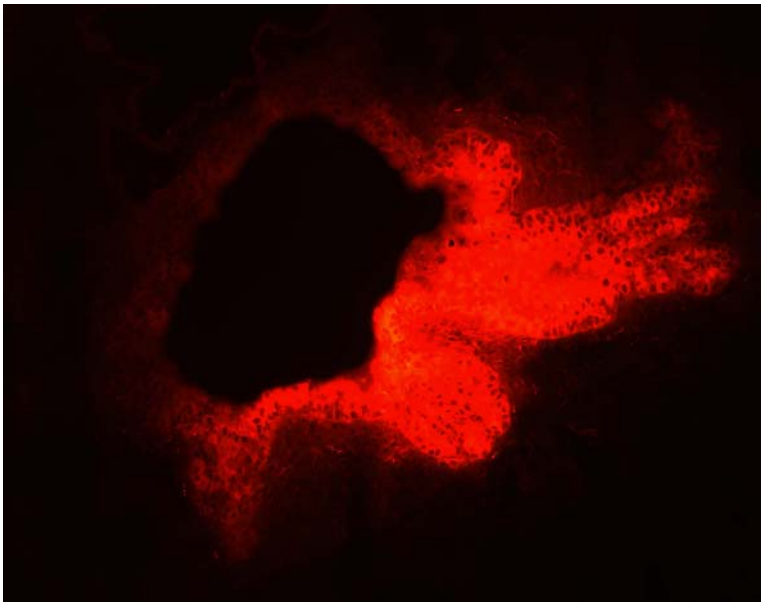

Figure 7. Example of rhodamin fluorescence in lung tissue. The fluorescent signal (red) of rhodamine in lung tissue is shown to be present in the epithelial lining of the airways. Magnification: 20x

Proof of concept
Different doses of spray dried hemin were administered to achieve HO-1 induction in lung tissue. Figure 8 shows a clear relationship between the dose of spray dried hemin and the level of HO-1 protein expression in lung

homogenate. Furthermore, inhalation of inert powder (spray dried inulin) containing sodium chloride did not result in increased expression of HO-1. HO-1 protein expression was found to be particularly increased in alveolar macrophages (figure 9). The airway epithelium stains faintly and shows no differences after hemin inhalation. The HO-1 expression as shown with HO-1-staining in the spray dried inulin exposed mice was comparable to control.

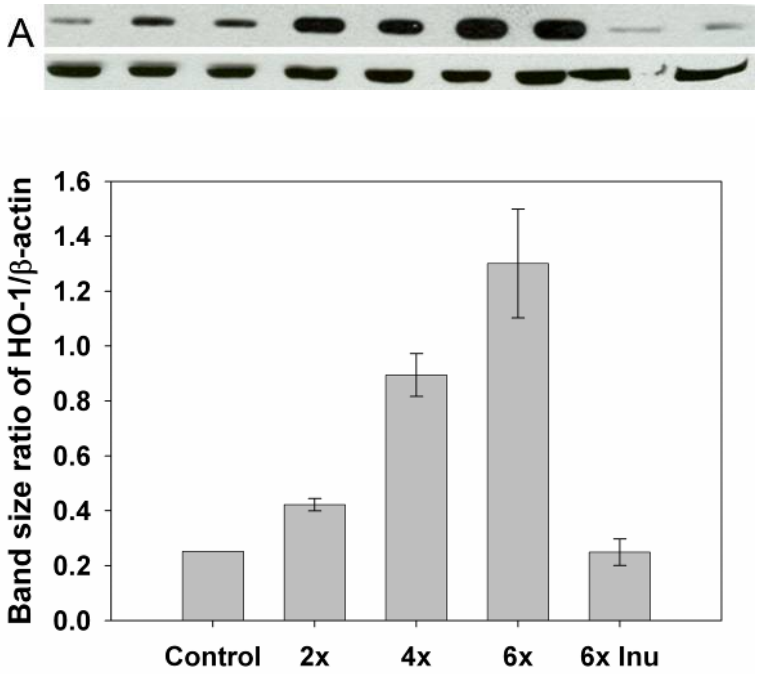

Figure 8. (A) HO-1 protein expression. Protein bands for HO-1 (top) and ß-actin (loading control, bottom) detected by Western blot analysis are shown; each band represents 1 mouse. From left to right: one control mouse that was not exposed to aerosol, 2 mice that were exposed to 2, 4 or 6 doses (2x, 4x and 6x) of spray dried hemin during 10, 20 or 30 minutes, respectively, and finally 2 mice that were exposed to 6 doses spray dried inulin (6x inu) during 30 minutes. **(B)** Ratio of the HO-1 and □-actin band size as obtained with Western blot for the different groups. Error bars indicate highest and lowest values.

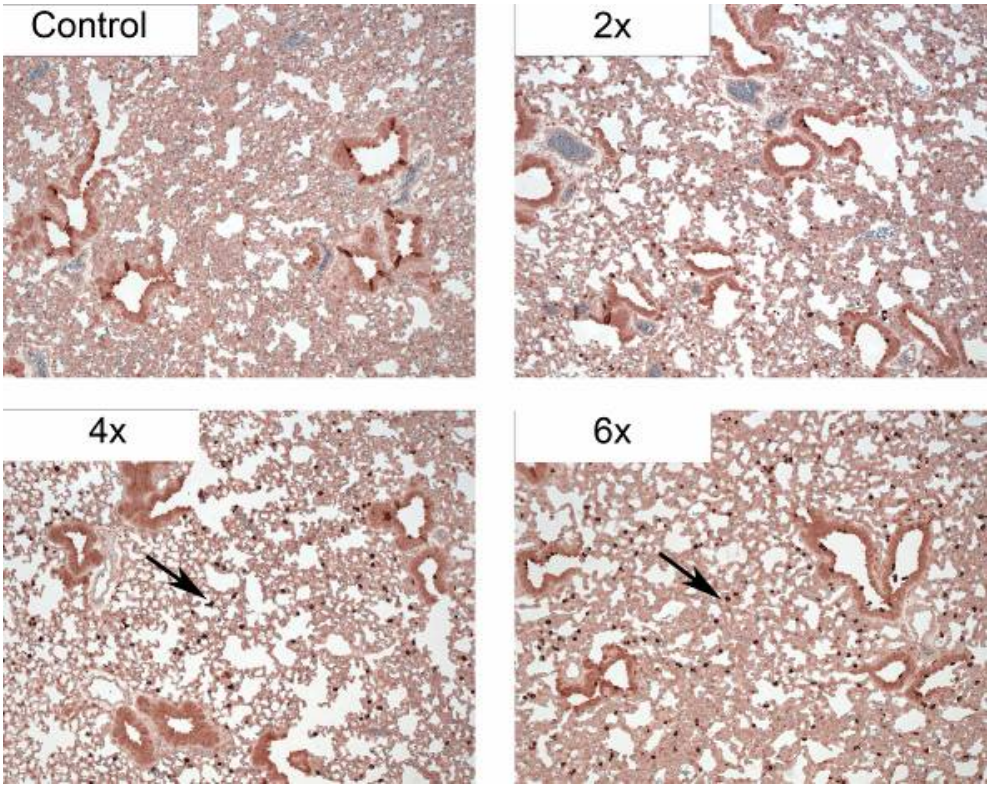

Figure 9. A representative picture of the HO-1 expression (dark red) in lung tissue of the control and after inhalation of 2, 4 and 6 doses of spray dried hemin is shown (25x magnification). Particularly, alveolar macrophages (indicated with an arrow) show an increased HO-1 expression after hemin inhalation. Magnification: 25x

The inflammatory side effects of spray dried hemin inhalation were studied by histological staining of leukocytes in lung tissue. Visual observation showed increased numbers of leukocytes surrounding the airways after inhalation of 4 and 6 doses of spray dried hemin, while in the control mouse and after inhalation of 2 doses of spray dried hemin this was not visible (figure 10).

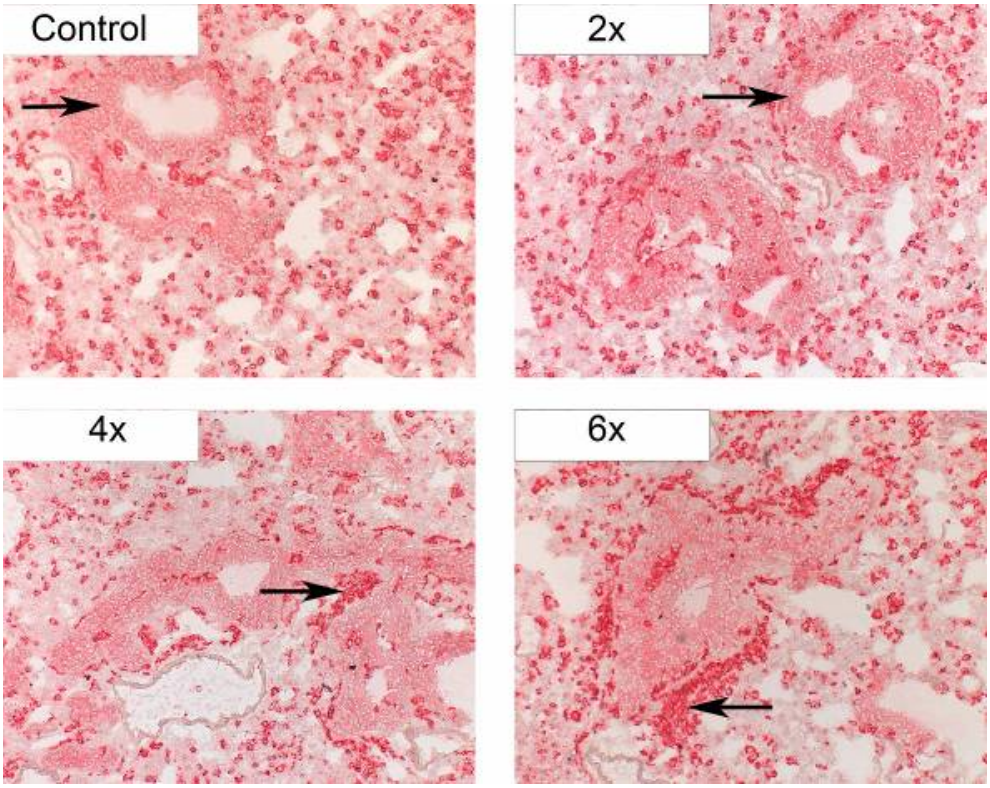

Figure 10. Leukocytes expressed in lung tissue. A representative picture of the leukocyte expression (CD45 positive cells) in lung tissue of the control and after inhalation of 2, 4 and 6 doses of spray dried hemin is shown. Arrows in top pictures indicate airways; arrows in bottom pictures indicate areas with increased numbers of leukocytes surrounding the airways. Magnification: 50x

DISCUSSION

Hemin, as shown in this study, can be formulated as a powder for inhalation by spray drying. Furthermore, spray dried hemin resulted in dose-dependent HO-1 induction after nose-only exposure of mice to the aerosol, showing that dry powder inhalation of hemin is possible and effective in inducing the HO-1 metabolic pathway.

Spray drying of hemin was performed from two solutions that had initial high pH values. An advantage of spray drying an ammonia solution that contains hemin is that ammonia evaporates during spray drying, resulting in pure hemin powder. In contrast, spray drying of the neutralized sodium hydroxide solution results in a powder that contains 37% w/w sodium

chloride and 63% w/w hemin. However, the latter solution results in a powder that dissolves much more rapidly than the pure spray dried powder. This is attributed to the presence of sodium chloride in the spray dried powder which improves wetting and increases the surface over which dissolution occurs. The technique may be beneficial for other poorly soluble compounds too, especially for those compounds that have a pH-dependent solubility.

Spray drying has become one of the standard techniques for the preparation of inhalation powders (25, 26). One of the advantages of spray drying over conventional techniques such as milling is the improved control over the particle size. From both the sodium hydroxide and ammonia solution the obtained powders were in the appropriate size range for inhalation, as measured with laser diffraction and cascade impactor analysis.

Clearly, the sodium chloride in the formulation did not increase HO-1 expression as was shown by the similarity in band size of HO-1 expression of the inulin control, after 6 doses, and the control that did not receive any aerosol.

The suitability of the Twincer-based aerosol box for pulmonary administration in freely breathing mice was demonstrated in this study. The *in vitro* laser diffraction measurements showed that an aerosol consisting of particles suitable for inhalation is present in the aerosol box for at least 5 minutes. Moreover, the in vivo study with rhodamin B showed that the aerosol reached all parts of the lung. It should however be realized that the individual mouse receives only a small fraction, about 0.02%, of the total dose in the Twincer. The major limitation in this setup is the relative small volume that is inhaled by mice.

One study exists where inhalation of hemin in human subjects has been investigated (13). However, the dose in our study is much higher than the dose used in the human study. The nebulized dose in the human study was 2 ml of 10^{-4} M hemin, which is equivalent to 1.6 μg/kg for a person of 80 kg. The lowest dose in our study is estimated to be 171 μg/kg, which corresponds to at least 100 times the dose used by Horvath *et al.* (13).

Since there was no information available on the dose-effect relationship of hemin we exposed mice to different doses. The dose escalation in our study was thus 171, 342 and 523 μg/kg. The relatively high doses that the mice inhaled led to concerns about the toxicity of hemin since it has been reported that hemin possesses oxidative properties especially at high

doses which is due to excess iron (27). Ambiguous effects have been ascribed to hemin (28). In moderate amounts and bound to proteins it is an essential element in various biological processes (10, 29). Large amounts of hemin can be toxic by mediating oxidative stress and inflammation, which is mainly due to release of iron (27).
The higher doses indeed led to infiltration of leukocytes into the airways. This inflammatory cell recruitment could be the result of increased production of inflammatory mediators by epithelial cells in response to toxic effects of the increasing dose of spray dried hemin. This possibility is in agreement with other *in vitro* and *in vivo* studies showing increased adhesion molecule expression and tissue infiltration of leukocytes after hemin administration to vascular cells (30, 31). On the other hand, we found that 171 µg/kg hemin did not result in increased numbers of inflammatory cells. However, inhalation of this dose of spray dried hemin was sufficient for a clear increase in HO-1 expression in lung tissue, indicating that this was a safe and effective dose of spray dried hemin.
To conclude, we presented a novel preparation of a hemin formulation by spray drying and showed proof of concept of the hemin formulation and a new pulmonary administration technique. The substance, hemin, induced the HO-1 expression in a dose dependent manner. Future studies investigating the utility of inhaled hemin in treating disease states are warranted.

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CHAPTER 8

Concluding remarks and future perspectives

CONCLUDING REMARKS

Over the past decades there has been a growing interest in the development of biopharmaceuticals as inhalation products, not only to obtain a local therapeutic effects but also to obtain a systemic absorption. As a consequence, pulmonary drug administration is nowadays connected to various therapeutic treatment modalities. Drug inhalation is no longer solely applied for treatment of local lung diseases, but its scope has widened to treat systemic diseases, to evoke a systemic effect without systemic absorption (e.g. vaccination) or to obtain systemic and local levels to treat for instance tuberculosis or cystic fibrosis.
Those biopharmaceuticals that are suited for inhalation can be formulated as a liquid, that is aerosolized by means of auxiliary energy at administration, or, they can be formulated as a powder for inhalation which is aerosolized upon inhalation by utilizing the patients' inspiratory force. Dry powder inhalation is the preferred means of administration for most patients as electricity or pressurized air are not needed. In addition, the short administration time, the better stability of the powder compared to a liquid formulation, the higher lung deposition, the more robust and reliable inhalation mode and size of the administration device are also advantageous..

In this thesis, we successfully formulated a number of biopharmaceuticals, each with different physico-chemical properties, as a dry powder for inhalation. Depending on the physical properties of the biopharmaceutical (e.g. stability, solubility, pK_a, molecular weight) a certain formulation strategy can be chosen. Relatively stable (small) peptides, such as cetrorelix or cyclosporine A, can be formulated as a powder for inhalation by using standard milling techniques and formulation as adhesive mixture or spherical pellet. More complex manufacturing processes and/or formulations may be needed when the biopharmaceutical is sensitive to chemical or physical degradation (e.g. rhDNase). So far the preferred manufacturing process seems to be spray drying because it is (next to freeze drying) the single process that has been successfully employed in commercial scale manufacturing for many years. Consequently, this process is well-known and well-described. A major disadvantage of the common freeze drying process is the fact that the obtained solid material has particulate properties that are unsuitable for inhalation and the material shows poor milling properties (e.g. high

tendency to stick to the walls of the equipment, moisture sensitivity, high tendency for crystallization). All other manufacturing processes (like spray freeze drying or supercritical drying) need further development and scale up and they are therefore expected to be more expensive. To protect chemically and/or physically sensitive biopharmaceuticals, we have demonstrated the versatility of sugar glass technology. Of the currently available excipients that can be used in sugar glass technology, inulin seems one of the best materials to meet stability demands: its high glass transition temperature allows storage at room temperature while the amorphous state is maintained. Other excipients, such as trehalose or sucrose tend to collapse at ambient conditions rendering them useless as inhalation products. Moreover, sugar glass technology in combination with spray (freeze) drying can be used for particle design. Particle size, shape and density can be designed to render material that in combination with the chosen inhaler provides the best possible aerosol. In addition, with sugar glass technology, the dissolution rate of poorly soluble active ingredients can be improved, which may improve their bioavailability.

Efficacy and safety research in animals are a relevant part of the pre-clinical development program of new drug products. For inhalation products it is almost impossible to mimic the pulmonary drug administration in humans. First, significant differences exist between man and animal regarding lung anatomy and breathing profile. Second, spontaneous inhalation through an inhaler is only rarely performed by most animals. Two powders for inhalation were tested in mice or rats, for which two different administration methods were used. The administration method of dry powder in mice was an aerosol chamber. The powder was aerosolized into the chamber by creating an inspiratory air flow through an inhaler. Mice were put in restrainers and attached to the aerosol chamber such that only their nose was exposed to the aerosol. The amount the mice inhaled depended on the aerosol concentration in the chamber and duration of exposure. With this model, different aerosol properties may be investigated when coupled with an appropriate method to determine deposition. The method used for aerosol administration to rats was intratracheal administration with the PennCentury insufflator. With the insufflator, aerosol properties are of less importance, yet accurate doses can be administered, which makes this method suitable for experiments where accurate dose administration is needed. Both

methods, nose-only exposure and insufflation, are complementary and considered essential models for inhalation product development.

FUTURE PERSPECTIVES

Biopharmaceuticals that will be evaluated as inhalation products should possess a true therapeutic advantage. When the only reason to develop an inhalation product is that it is "a more comfortable route of administration than the injection" the chances of market success are limited as was shown by the Exubera® case. Only in those cases where pulmonary administration offers a true therapeutic advantage, such as an increased efficacy or an improved safety, pulmonary biopharmaceuticals may become successful. Typical examples of such biopharmaceuticals are:

- biopharmaceuticals that can be used to treat local lung disease,
- biopharmaceuticals that evoke the best systemic therapeutic response after inhalation (e.g. vaccination), or
- biopharmaceuticals that cause serious side effects when administered via another route (e.g. injection)
- biopharmaceuticals that require rapid absorption, but for any reason cannot be injected (e.g. for hygienic reasons)

For each biopharmaceutical suited for inhalation a taylor-made manufacturing process and formulation is to be designed that meets general requirements, such as the pharmaceutical aspects as well as aspects related to efficacy, safety, patient compliance and health care costs. The technology to develop and manufacture biopharmaceuticals as powders for inhalation is nowadays available although the application of the technology is still rather limited.

One of the major research topics that may enable a broader use of this technology in the future is the understanding of the processes that occur during particle formation. With this knowledge, particles that are easily dispersible and demonstrate excellent deposition characteristics can be prepared. Drug product and administration device should be regarded as a single entity since not all formulations will be optimally aerosolized with just one inhaler; i.e. the best possible inhaler will not be suitable for all different formulations. The optimal result can only be reached when formulation and inhaler are optimized regarding the specific characteristics and interactions they have. Furthermore, computational

fluid dynamics and computational particle tracking will receive more attention in the inhalation field, since these technologies can speed up product development, both by indicating the optimal starting point for a development based on *in silico* simulations as well as through the generation of additional information on deviating performance during development.

Further refinement in animal models for pulmonary drug products is also anticipated but also urgently required. To appropriately test inhalation drug products, improved animal models are needed since the use of animal models is a prerequisite to substantiate the marketing authorization dossier. These models should be able to discriminate between different drug products in terms of aerosol behaviour and subsequent pharmacokinetics and –dynamics. In addition, the deposition characteristics would need to be analyzed. Improvements in methods to determine the deposition characteristics in laboratory animals are possible, for example by *in vivo* bioluminescence or fluorescence.

Safety of biopharmaceuticals is a key aspect as protein structures may change as a consequence of purification, production, storage or even use. Such changes may lead to unwanted immunological responses, even when patients are immune tolerant to the endogeneous protein. As at present the exact role of an altered protein structure is unknown, biopharmaceuticals are likely to be subjected to rigorous immunological profiling in animal studies, but also in humans.

Finally, many research efforts will be fuelled by various societal aspects related to the current state of the pharmaceutical industry and policies around it. The declining success of pharmaceutical research is emptying the industry's pipelines; this will result in fewer new drug products that enter the market. Those products that are promising should be brought to the market as fast as possible and in the end in the most optimal way possible, in order to cover for losses of unsuccessful development projects.
Tighter requirements from regulatory authorities address society's requirement for more efficient and safer medicines, preferably provided at lower costs. However, since safety and efficacy can only be improved through extensive research and optimized drug administration, possible

future cost savings can only be generated through significant investments in today's pharmaceutical research and development.

To conclude, this thesis demonstrates that many manufacturing processes and formulations are already available to design tomorrow's biopharmaceutical drugs as inhalation products.

CHAPTER 9

Summary

The rapid developments in molecular biology and biotechnology during the past decades have resulted in the successful introduction of a number of biopharmaceuticals (among which many therapeutic peptides and proteins) on the market. Biopharmaceuticals are a special class of medicines in the sense that, in general, they are chemically and physically not stable. In addition, biopharmaceuticals rely on a certain (three dimensional) folding structure for their efficacy and safety. Retaining the folding structure is essential to develop efficacious and safe drug products of these substances.

Of all biopharmaceuticals, a number may well be suited for inhalation. The objective of the inhalation can be to exert either a local or systemic action, or both. Various device types are available for inhalation. The aerosol can be created from liquids or dry powders. Nebulization is not patient friendly; it is time consuming, requires electricity or pressurized air and the efficacy of aerosolisation and lung deposition are poor which is associated with unnecessary wastages. On the other hand, dry powder inhalation typically needs less than a minute for completion and the efficiency of aerosol production and lung deposition are relatively high.

As biopharmaceuticals are often manufactured in solutions, a drying step is required to make them suitable for powder inhalation. Especially those biopharmaceuticals that depend on their tertiary structure for activity, require stabilization during drying and storage. Excipients like sugars seem suitable for this purpose. They immobilize the molecules and offer the possibility to replace the hydrogen bonds between drug and solvent.

Several drying methods are available and a number of these methods have been investigated in this thesis: freeze drying, spray-freeze drying and spray drying. Other techniques like supercritical fluid extraction may be suitable too. Spray-freeze drying gives a solid powder as end-product. By appropriate settings of the process conditions a powder with the desired aerodynamic particle size may be obtained. Spray drying also provides the possibility to produce a powder within the desired size range.

In this thesis we investigated the formulation of several peptides and proteins as powder for inhalation. Furthermore, a “proof-of-concept” study was performed with several products. We examined a selection of peptides and proteins, with different properties in terms of hydrophilicity and molecular size. Finally an innovative concept, based on the inhalation

of a small molecule that induced the endogenous production of a protein (an enzyme) was investigated.

In **Chapter 2**, three manufacturing methods were evaluated for their ability to produce a dry powder inhalation of cetrorelix, a hydrophilic peptide that consists of 10 amino acids. Cetrorelix was milled, spray dried and spray-freeze dried. The fragile structure of the spray-freeze dried powder made it unsuitable for processing to an adhesive mixture. The three methods resulted in particles with different morphologies, although all were in the appropriate size range for inhalation. The aerosolization behavior of the mixtures with milled and spray dried cetrorelix was tested in two commercially available devices: a capsule-based inhaler and the Novolizer. The capsule-based inhaler uses the rotation and vibration of the capsule for deagglomeration, while the Novolizer utilizes a cyclone-based principle that uses impact forces for deagglomeration. When the adhesive mixtures of milled and spray dried cetrorelix were dispersed with the capsule based inhaler and the Novolizer, both powder mixtures had appeared to have a higher fraction of cetrorelix on the third and fourth stages of the cascade impactor when dispersed with the Novolizer. The morphology of the spray dried powder turned out to be better suitable for aerosolisation than that of the milled material in both inhalers. This investigation showed that the adequate development of inhalation systems requires a dual approach in which the specific combination of both the formation and device should be optimized.

In **Chapters 3 and 4** the hydrophobic peptide Cyclosporine A was investigated. Cyclosporine A is a cyclic undecapeptide, consisting of 11 amino acids. The inhalation of this drug could be used to prevent rejection of lung transplants. The utility of solid dispersion technology to manufacture a Cyclosporine A powder for inhalation was investigated. The solid dispersions were produced from a co-solvent system of water and *Tert*-butyl alcohol (TBA) using inulin with a degree of polymerization of 23 as carrier. After spray-freeze drying, a homogeneous powder Cyclosporine A and inulin with a high dissolution rate is produced. Fourier transform infrared spectroscopy (FTIR) was used to evaluate how Cyclosporine A was incorporated in the matrix. It was demonstrated that at increasing drug loads, more Cyclosporine A was present in a hydrophobic environment, indicating that more Cyclosporine molecules

were in close contact with each other, and possibly formed small clusters of even particles within the matrix. The formation of clusters might explain why powders with higher drug loads dissolved slower.
Spray-freeze drying results in low density particles with a very high specific surface area of more than 100 m^2/gr. In spite of their large geometric size, the particles had still an aerodynamic size within the desired range of 1 to 5 µm, which is explained by the high porosity of the particles. The aerodynamic particle size was appropriate for inhalation, with a mass median aerodynamic diameter of 3.39 µm. The solid dispersion with 50% w/w CsA had the appropriate quality attributes for inhalation; a high dissolution rate and excellent aerodynamic behaviour.

Chapter 4 describes the *in vivo* properties of the Cyclosporine A powder for inhalation in rats. **Chapter 4A** focuses on pharmacokinetics and efficacy in preventing lung rejection of the Cyclosporine A powder for inhalation (iCsA) in a rat model. The pharmacokinetics of 10 mg/kg iCsA and Neoral, the oral commercial product containing Cyclosporine A, were studied. Although iCsA and Neoral resulted in comparable systemic exposure as determined by the area under the curve of the plasma concentration versus time curve and the same kidney exposure, iCsA had 109-fold higher lung tissue levels than Neoral.
In the proof-of-concept study, the rat model demonstrated acute rejection within 8 days as a result of the incompatibility of the donor and recipient. iCsA was already effective at a lower dose than Neoral. However, in the used iCsA dose (5 mg/kg) local reactions in the lungs of healthy, non-transplanted, rats were observed. Therefore, in **Chapter 4B** the effects of lower doses were studied and compared with the effects to placebo. At a dose of 0.8 mg/kg, iCsA seemed to cause no different effect as a placebo.
Although in Chapter 4A we demonstrated that doses lower than 5.0 mg/kg would not prevent rejection in the animal, the 0.8 mg/kg dose is relevant to humans, since it was described before that in man lower inhaled doses were already effective. We anticipate to administer a dose of 6.25 mg CsA twice daily, which would correspond to approximately 0.1 mg/kg for a 60-kg person. Even at such a dose, the local lung tissue levels of iCsA would increase substantially, while the contribution to systemic levels would be relatively small which reduces the risk of associated

nephrotoxicity. Hence, the efficacy of the treatment could possibly be improved in patients that otherwise have no therapeutic options left.

Chapter 5 describes a study into the possibilities of formulating a large protein, recombinant human deoxyribonuclease I (rhDNase) as a powder for inhalation, whereas **Chapter 6** provides a pharmacoeconomic analysis of such a product.
To formulate the protein we evaluated different excipients, processes and process conditions. In addition, the efficacy of the most suitable formulation was demonstrated with an *ex vivo* model. The excipient selection was performed by freeze drying deoxyribonuclease I in the presence of sucrose, trehalose and two types of inulin. Sucrose was found to be unsuitable as stabilizer, whereas trehalose appeared to be the best stabilizer. Furthermore, spray drying and spray-freeze drying were compared as potential manufacturing processes under different process conditions. Spray drying was the best suited process, as particles with a mass median aerodynamic diameter of 2.3 µm could be produced. Of the tested compositions only the inulin (DP 23) formulation could be stored at ambient conditions. The inhalation characteristics of this formulation were also good, with a fine particle fraction (< 5 µm) of approximately 40%. Therefore, we evaluated the visco-elastic properties of sputum from CF patients before and after applying the rhDNase/inulin DP23 formulation. The rhDNase powder for inhalation significantly decreased both elasticity and viscosity. We concluded that even larger proteins can be formulated as a powder for inhalation. Careful screening of excipients and processes may lead to the best possible combination, resulting in an inhalation powder with the best possible properties in terms of activity, storage stability and aerodynamic particle size.

Chapter 6 describes the pharmacoeconomic aspects of nebulization versus dry powder inhalation of rhDNase, a key product in the management of cystic fibrosis. The potential advantages of dry powder inhalation are *i)* improved compliance to therapy, *ii)* improved deep lung deposition, *iii)* no need for electricity of pressurized air, *iv)* reduced administration time and *v)* no need to cool the rhDNase solution. These benefits may among others lead to less exacerbations and less hospitalizations. The costs of rhDNase nebulization were estimated at €19.110 for a Life Year Gained (LYG), while the cost for a LYG of dry

powder inhalation was estimated at €12.100, which is 40% less than nebulization. This demonstrates that it may be useful to investigate already in an early stage of development what the pharmacoeconomic impact of certain formulation decisions may be.

In **Chapter 7** a highly innovative biopharmaceutical type of invention was explored. The small organic molecule, hemin was pulmonary administered in order to induce the endogenous production of a protein heme oxygenase I (HO-1). Such a approach would omit the difficult formulation of a protein for inhalation. HO-1 is a protein of interest because this enzyme is decreased in COPD patients compared to healthy subjects.
In the formulation process an alkaline solution of the poorly soluble hemin was neutralized immediately before spray drying, to yield a product containing hemin and sodium chloride. A new administration device for administering inhalation powders to small laboratory animals, using the natural breathing pattern, was also developed. It was demonstrated that HO-1 could be induced in a dose-dependent manner with inhaled hemin. This study showed that also with small organic molecules a biopharmaceutical intervention may be possible. Furthermore a novel method to allow preclinical testing of inhalation powders was introduced. This method is unique as it uses the inspiratory air flow of the animals, instead of “forcing” the powder into the lungs by insufflation. This opens the possibility to study the deposition characteristics of different powders in the preclinical setting.

In conclusion, the selected therapeutically active compounds could all be formulated as a powder for inhalation. Depending on the properties of the molecule, taylor-made formulation strategies could be employed to guarantee the structural integrity during drying and subsequent reconstitution. In some cases the pulmonary administration of a small molecule that induces the endogenous production of the therapeutic protein may be a good alternative, since it simplifies the formulation and less critical parameters need to be taken care of. As powders for inhalation rely on their aerodynamic properties for deposition and efficacy, there is a need to test the powders on these properties in a non-invasive manner in laboratory animals. In this thesis we used the insufflation and nose-only exposure approach. Insufflation is more suited for accurate dose-effect studies, while nose-only exposure can be used to

deliver a proof-of-concept, in which dose precision is less critical. In such a system, various formulation attributes (size, density) may be tested to find the best inhalable powder.

APPENDIX

Pilot study of Cyclosporine A dry powder inhalation in lung transplant patients with Bronchiolitis Obliterans Syndrome

Positief beoordeeld door
Medisch Ethische Toetsingscommissie Universitair Medisch Centrum Groningen

SAMENVATTING

Longtransplantatie is een behandeling welke voor de meeste indicaties overlevingswinst betekent. Eén van de complicaties is chronische afstoting. Dit is een complicatie welke zeer ernstig is en waarvoor tot op heden geen farmacotherapeutische opties voorhanden zijn. Het eindstadium van chronische afstoting is ofwel re-transplantatie of overlijden.
Ciclosporine A is een geneesmiddel wat veel gebruikt wordt ter voorkoming van afstoting. De belangrijkste bijwerking van ciclosporine is echter nefrotoxiciteit waardoor de dosis verlaagd moet worden. Op den duur leidden de dosisaanpassingen tot een grotere kans op chronische afstoting. Met andere woorden; de overlevingswinst bij longtransplantatie wordt geremd door de toxiciteit van de behandeling.
Lokale toediening van ciclosporine A, middels inhalatie, is dan ook een farmacotherapeutische optie voor het behandelen van chronische afstoting omdat de systemische circulatie minimaal wordt belast met ciclosporine en omdat lokaal hoge spiegels kunnen worden gehaald.
In dit protocol wordt een studie beschreven waarin 5 tot 7 patienten met matige tot milde (bronchiolitis obliterans syndroom stadia 1 en 2) chronische afstoting behandeld gaan worden met droog poeder inhalatie van ciclosporine gedurende een periode van zes maanden. De formulering en de inhaler zijn ontwikkeld door de werkgroep Farmaceutische Technologie en Biofarmacie van de Rijksuniversiteit Groningen. De primaire vraagstelling in dit onderzoek is of droog poeder inhalatie van ciclosporine A effectief is in de behandeling van chronische afstoting, welke niet behandelbaar is met de huidige farmacotherapeutische opties, in patiënten met een longtransplantaat? Daarbij wordt de nierfunctie ook gemeten ten tijde van de studie om te onderzoeken of de eventuele werkzaamheid niet gepaard gaat met nefrotoxiciteit.
Voor deze studie zijn weinig extra procedures nodig, behalve een éénmalige inhalatie van radioactief materiaal om de efficientie van de inhalatie te bepalen, het innemen van de studiemedicatie en de bepaling van ciclosporinebloedspiegels. De patienten in de studie mogen hun huidige immuunsuppressieve therapie blijven gebruiken om te waarborgen dat er zo weinig mogelijk afstoting plaatsvindt.
Het voorliggende protocol is het eerste in een reeks van onderzoeken. Deze studie is erop gericht om informatie te krijgen over de efficientie van de inhalatie, de hoogte van ciclosporine bloedspiegels na inhalatie en

de effectiviteit van droog poeder inhalatie van ciclosporine op de behandeling van patienten met chronische afstoting van het longtransplantaat. Het is de bedoeling dat dit onderzoeksgeneesmiddel uiteindelijk uitgelicenseerd wordt aan een geinteresseerd bedrijf.

INTRODUCTIE EN RATIONALE

Longtransplantatie in het algemeen

Longtransplantatie wordt vanaf midden jaren 80 van de vorige eeuw succesvol toegepast. De indicatie van longtransplantatie is een eindstadium longziekte, zoals cystische fibose, longfibrose en pulmonale hypertensie. De prognose van dergelijke patiënten zonder transplantatie is zeer beperkt, in het algemeen is de overleving minder dan twee jaar. Met transplantatie wordt door de International Society for Heart and Lung Transplantation (ISHLT) een mediane overleving van 4,4 jaar gemeld. In Groningen is de mediane overleving zelfs 7,5 jaar. Dit betekent dat bij de meeste indicaties het uitvoeren van longtransplantatie overlevingswinst oplevert. Daarnaast treedt na transplantatie een normalisering van de longfunctie op en neemt de kwaliteit van het leven significant toe. [1]

Therapie van longtransplantatie

Om een longtransplantaat succesvol te laten functioneren is het remmen van het immuunsysteem van de ontvanger onvermijdelijk. Dit wordt in de praktijk gedaan met onder meer systemische toediening van zogenaamde calcineurine-blokkers als ciclosporine (CsA) en tacrolimus (Tac). Het succes van longtransplantatie en transplantatie in het algemeen is grotendeels te danken aan deze middelen. CsA en Tac worden veelal in combinatie gegeven met nucleosideremmers (azathioprine en mycofenolaat-mofetil) en corticosteroïden.

Omdat de calcineurine-blokkers niet universeel effectief zijn in een volledige preventie van acute longtransplantaatrejectie (AR) en de daaraan gerelateerde chronische dysfunctie van het longtransplantaat (Bronchiolitis Obliterans Syndroom; BOS) blijft er risico bestaan op afstoting. Zo wordt de kans op minimaal 1 AR episode na longtransplantatie variabel beschreven (30 – 80%). Het optreden van BOS, al dan niet in combinatie met daaraan gerelateerde pulmonale infecties, de belangrijkste risicofactor voor de lange termijn overleving na longtransplantatie. Om BOS zoveel mogelijk te voorkomen zijn hoge doses calcineurine-blokkers nodig. De systemische toediening resulteert in een

aantal nadelen. Er is een toegenomen risico op infectie en maligniteit en de behandeling heeft nefro- en neurotoxiciteit tot gevolg. Het optreden van hypertensie door nierinsufficiëntie (ontstaan door behandeling met hoge doses calcineurine-blokkers) beperkt de overleving en kwaliteit van het leven sterk. De dagelijkse praktijk is dan ook dat de dosering van calcineurine-blokkers wordt aangepast op geleide van de mate van afstoting en de nierfunctie. Het zoeken naar de balans in werking (voorkomen/behandelen BOS) en bijwerking (optreden niertoxiciteit) beperkt de therapeutische effectiviteit op de lange termijn. De prevalentie in het optreden van BOS bedraagt 20% en 70% na respectievelijk 2 en 7 jaar na longtransplantatie (www.ishlt.org). BOS en BOS gerelateerde infectie zijn verantwoordelijk voor ongeveer 50% van de late mortaliteit. Hierom is preventie en behandeling van BOS één van de belangrijkste uitdagingen op het gebied van longtransplantatie.

Pulmonale toediening van calcineurine-blokkers

Eén van de mogelijkheden om de systemische bijwerkingen van calcineurineblokkers te vermijden en tegelijkertijd de lokale dosis in het getransplantanteerde orgaan te verhogen is inhalatie. Dankzij deze voordelen zou inhalatie bij uitstek geschikt zijn om niet behandelbare chronische afstoting alsnog te behandelen door de hogere lokale dosis. Dit idee is niet nieuw, de Pittsburgh-groep (hoofdonderzoeker Iacono) is al ongeveer 15 jaar bezig met het vernevelen van een CsA-oplossing. In het kort zijn dit enkele belangrijke conclusies:

- Verneveling van ciclosporine leidt in vergelijking tot systemische toediening tot hogere concentraties in longweefsel [2]
- Verneveling geeft minder afstoting-gerelateerde inflammatie [3]
- Er is een correlatie tussen de remming in afname van FEV_1 met de pulmonale depositie van ciclosporine; 5 mg perifere depositie lijkt het cut-off point te zijn [4]
- In een case control studie werd bij 39 patienten met Bronchiolitis Obliterans na verneveling van ciclosporine een significante overlevingswinst (1 tot 2 jaar) aangetoond ten opzichte van twee retrospectieve controle groepen (100 en 59 patienten). [5]

De hierboven beschreven behandeling met verneveling van ciclosporine is altijd gesponsord door Novartis. Novartis is voor de hier voorgestelde studie meerdere malen geraadpleegd over samenwerking; dit was

juridisch gezien niet mogelijk daar de rechten met betrekking tot het product (Pulminiq) zijn verkocht aan Chiron. Chiron wilde vervolgens niet samenwerken omdat het product alleen op de Amerikaanse markt zal worden gebracht. De status van het product is dat men wacht op marktautorisatie van de FDA. Tot op heden heeft verneveling van ciclosporine een NDA (investigational New Drug Application – 50-799 - [6], antwoord FDA [7], beide bijgevoegd als bijlage bij dit protocol) status. Uit deze gegevens blijkt dat verneveling van ciclosporine een effectieve en aantrekkelijke optie is voor de behandeling van chronische afstoting welke niet meer behandelbaar is met de huidige therapeutische opties. De FDA heeft aan de hand van NDA 50-799 enkele vragen gesteld aan een advisory committee gesteld [7], hier is een transcriptie van te vinden op internet: (http://www.fda.gov/ohrms/dockets/ac/05/transcripts/2005-4135T1.pdf).

Verneveling is echter een methode die niet praktisch is. De duur van toedienen is ongeveer 20 tot 30 minuten en dan bereikt slechts 10% van de dosis de luchtwegen. Daarnaast is perslucht of stroom nodig om de druppeltjes te creëren. Dit beperkt de mobiliteit van de patient. Van droog poeder inhalatie is bekend dat de dosis die de luchtwegen bereikt tot 4x zo hoog is in vergelijking met verneveling. Daarnaast duurt de inhalatiemanoeuvre veel korter. Droog poeder inhalatie zou dus een betere optie zijn. In de tijd dat Iacono en cs. begonnen met verneveling was er geen geschikte droog poeder formulering van ciclosporine beschikbaar vanwege de fysisch chemische eigenschappen van ciclosporine. Ciclosporine is namelijk slecht wateroplosbaar (slechts 3 mg/L) en derhalve zou puur microniseren resulteren in een nauwelijks werkzaam poeder. De werkgroep Farmaceutische Technologie en Biofarmacie (Farmacie, faculteit der Wiskunde en Natuurwetenschappen, Rijksuniversiteit Groningen) hebben ciclosporine echter geformuleerd als een amorf suikerglas in combinatie met inuline. De zo onstane formulering heet in vakjargon een solid dispersion. Solid dispersions worden vaak toegepast om de oplossnelheid te verbeteren van slecht wateroplosbare stoffen. Het is gebleken dat ciclosporine geformuleerd als solid dispersion sneller oplost dan puur ciclosporine. Voorts is gebleken dat deze formulering zeer goede inhalatie-eigenschappen heeft.[8] (tevens bijgevoegd als bijlage) Deze formulering zou dan ook geschikt zijn voor de behandeling van patiënten welke een longtransplantatie hebben

ondergaan en niet behandelbare (met de huidige farmacotherapeutische opties) chronische afstoting vertonen. Het vernieuwende binnen deze mETC aanvraag is dat er droog poeder inhalatie wordt gebruikt in plaats van verneveling. Dit zal de therapietrouw moeten verbeteren alsmede de effectiviteit. De hulpstof is tevens minder schadelijk (inuline versus propyleenglycol) en is de dosis vele malen lager (dagelijks 12,5 mg vs 150 mg) dankzij verhoogde efficientie. Dit zijn twee belangrijke argumenten waarom het onderzoeksgeneesmiddel in het voorliggende protocol een beter product is dan de verneveling. Daarnaast is er voor dit geneesmiddel een inhaler ontworpen, genaamd de twincer. Het principe waarmee de deeltjes in de juiste deeltjesgrootte vrijkomen uit de inhaler is eerder gebruikt[9-12] en deze inhaler is een doorontwikkeling daarvan.[13] De inhaler bestaat uit vier gedeelten: een bodemplaat met daarin ruimte voor de blister en het desintegratieprincipe, een middenplaat met daarin het tweede gedeelte van het desintegratieprincipe en de topplaat met het mondstuk. Daarnaast uiteraard nog de blister (zie foto's)

De Twincer, links de gemonteerde versie, rechts de vier onderdelen los (rechts de onderplaats, links de bovenplaat)

Omdat met inhalatie de systemische bijwerkingen in principe kunnen worden geminimaliseerd met gelijkblijvende effectiviteit is het de bedoeling om uiteindelijk te onderzoeken in hoeverre droog poeder inhalatie van ciclosporine ingezet kan worden als standaard behandeling van patiënten met longtransplantatie. Voordat er genoeg data beschikbaar is om aan te tonen dat dit mogelijk is, zullen verschillende onderzoeken plaats moeten vinden. Het voorliggende protocol vormt de

eerste stap binnen dit geheel. Blijkt dat chronische afstoting succesvol behandelt kan worden met droog poeder inhalatie van ciclosporine bovenop de standaardtherapie zonder extra systemische bijwerkingen dan kan de volgende stap gezet worden. Is de behandeling wel effectief dan zou een volgende studie antwoord moeten geven op de vraag in hoeverre inhalatie van ciclosporine effectief is in het behoud van longfunctie terwijl de orale immuunsuppressieve therapie wordt afgebouwd. Indien blijkt dat deze studie niet effectief is in het behandelen van chronische afstoting dan nog is vervolg onderzoek mogelijk op het voorkomen van nierschade door systemische immuunsuppressie of op de lengte van een chronische afstoting vrij interval.
Het is de bedoeling dat dit product uiteindelijk zal worden uitgelicenseerd aan een geïnteresseerd bedrijf welke dit dan op de markt zou kunnen gaan brengen.

Samenvattend over de wetenschappelijke vraagstelling voor deze studie: is droog poeder inhalatie van ciclosporine A effectief in de behandeling van chronische afstoting, welke niet behandelbaar is met de huidige farmacotherapeutische opties, in patiënten met een longtransplantaat?

DOELSTELLINGEN

De primaire en de secundaire doelstelling zijn - inherent aan het onderzoeksgeneesmiddel en route van toediening - onlosmakelijk met elkaar verbonden. Met het onderzoeksgeneesmiddel in dit voorliggende protocol wordt getracht een betere behandeling met in ieder geval vergelijkbare systemische bijwerkingen (hopelijk, en naar verwachting, minder op o.a. de nier) en betere lokale verdraagzaamheid dan eerdere formuleringen van geïnhaleerd ciclosporine.

Primaire doelstelling

De primaire doelstelling van dit onderzoek is het effectief onderdrukken van chronische afstoting van de getransplanteerde long. Dit wordt bewerkstelligd door inhalatie van ciclosporine als droog poeder als extra therapeutische interventie, bovenop de standaardtherapie. De hypothese is dat door de lokale toediening van ciclosporine hogere lokale spiegels worden bereikt in vergelijking met systemische toediening waardoor het immuunsysteeem krachtiger wordt onderdrukt en dat daardoor de chronische afstoting sterk wordt gereduceerd.

Secundaire doelstelling

De secundaire doelstelling is dat de primaire doelstelling wordt gehaald zonder extra nefrotoxiciteit. Omdat geneesmiddelen welke op het calcineurinesysteem werken vaak nefro- en neurotoxisch zijn, kan theoretisch gezien de therapeutische interventie met geïnhaleerd ciclosporine in deze proefopzet aanleiding geven tot extra nefrotoxiciteit. Dit zou kunnen ontstaan doordat ciclosporine voor een deel door de long wordt geabsorbeerd en in de systemische circulatie terechtkomt. De verwachting is dat de hoeveelheid geabsorbeerd ciclosporine dermate laag is dat dit geen nefrotoxiciteit met zich meebrengt.[14]

Andere doelstellingen

Om het effect van geïnhaleerd ciclosporine te kunnen correleren aan de mate van afstoting is het van belang om naast de therapeutische interventie een studie uit te voeren waarin wordt onderzocht hoeveel van het poeder op een bepaalde plaats in de longen terechtkomt. Dit zal worden bepaald met een depositiestudie in samenwerking met nucleaire geneeskunde van het UMCG hetgeen onderdeel is van dit protocol (zie ook §8 Radiofarmacon voor depositie).

STUDIE-OPZET

De studie is als een descriptieve interventiestudie opgezet. Voor dit format is gekozen omdat er binnen de kliniek van het UMCG de laatste jaren een afname is opgetreden in het aantal patiënten die chronische afstoting ervaren en voldoen aan de inclusie- en exclusiecriteria. Naar verwachting zijn dit transversaal 5 tot 7 patiënten. Omdat chronische afstoting een ernstige complicatie met sterk beperkte levensverwachting is en omdat er geen farmacotherapeutische opties meer voorhanden zijn voor deze aandoening is gekozen voor een descriptieve, interventie studie. Dit betekent dat 5 tot 7 bekende patienten die voldoen aan in- en exclusiecriteria behandeld zullen worden met het onderzoeksgeneesmiddel.

STUDIE POPULATIE

Doelgroep

De interventie met droog poeder inhalatie van ciclosporine is in algemene zin bedoeld voor patiënten die een longtransplantaat hebben ontvangen en heeft als therapeutische indicatie het voorkomen en onderdrukken van

chronische afstoting van de getransplanteerde long. De patiëntenselectie voor deze studie is echter strikter, binnen deze studie gaat het om patiënten met een getransplanteerde long die reeds een bepaalde mate van chronische afstoting hebben ontwikkeld. Voor deze groep patiënten zijn er geen (farmaco-)therapeutische opties meer voorhanden en daarom neemt dit onderzoeksgeneesmiddel een potentieel belangrijke plaats in bij de behandeling van chronische afstoting.

Inclusiecriteria

- Leeftijd: ouder dan 18 jaar
- Toestemming middels Informed Consent
- Primaire longtransplantatie met tacrolimus als onderhoudsimmunosuppressivum
- Bronchiolitis Obliterans Syndroom (BOS) stadium 1 tot en met 3: FEV_1 <80% ten opzichte van basisniveau
- Minimaal 3 maanden na de laatste gebruikelijke interventie wegens BOS
- Verslechteren klinisch beeld (FEV_1) na laatste gebruikelijke interventie wegens BOS
-

Exclusiecriteria

- Leeftijd: jonger dan 18 jaar
- Niet wilsbekwaam
- Huidige behandeling bevat ciclosporine
- Bronchiolitis Obliterans Syndroom stadium 0: FEV_1>80%
- Glomerulaire filtratiesnelheid lager dan 30 ml/min
- Klinisch stabiele patiënten
- Chronische luchtweginfecties in het kader van BOS
- Zwangerschap

Aantal patiënten

Het maximaal aantal haalbare patiënten binnen de kliniek zal worden geincludeerd in deze pilot studie. Voorlopige analyses wijzen uit dat dit een populatie betreft van 5 tot 7 patiënten welke voldoen aan de in- en exclusiecriteria. De ervaring is dat deze patienten zeer gemotiveerd zijn om te participeren in onderzoek derhalve is de verwachting dat 5 tot 7 patienten geincludeerd kunnen worden in deze pilot studie.

THERAPEUTISCHE INTERVENTIE

Onderzoeksgeneesmiddel

De interventie bestaat uit inhalatie van CsA als droog poeder, geformuleerd als een solid dispersion (definitie solid dispersion: een groep geneesmiddelvormen waarin één of meer actieve stoffen gedispergeerd zijn over een matrix van een biologisch inerte stof). Deze solid dispersion wordt verkregen met sproeivriesdrogen. De formulering van CsA in inuline wordt in blisters uitgevuld. De blisters worden vervolgens in speciaal daarvoor ontwikkelde single-use inhalatoren geplaatst. De blisters zitten daarna vast in de inhalator en kunnen niet meer worden verwijderd. De inhalator is dan onderdeel van het geneesmiddel en heeft als zodanig dan ook geen type goedkeuring nodig. De patient dient twee inhalaties van 12,5 mg per inhalatie tot zich te nemen. Een meer gedetailleerde omschrijving is te vinden in §7, “onderzoeksgeneesmiddel”.
In dit onderzoek wordt geen placebo meegenomen omdat de patient in de gekozen studie opzet zijn eigen controle is.

Gebruik van co-medicatie

Het gebruik van het onderzoeksgeneesmiddel als rescue therapie is bovenop de bestaande therapie bij chronische afstoting. Dit wordt gedaan om een maximaal effect te bewerkstelligen in de behandeling van chronische afstoting. Het gebruik van co-medicatie in deze studie is dan ook zonder limitaties toegestaan, daar co-medicatie een belangrijke component is voor de effectieve behandeling van longtransplantatie patiënten. Het gebruik van geneesmiddelen wordt overigens oplettend gevolgd door de longtransplantatie-afdeling. Voor de bloedspiegelbepaling is echter noodzakelijk dat de patienten niet op andere wijze ciclosporine krijgen toegediend dan via inhalatie.

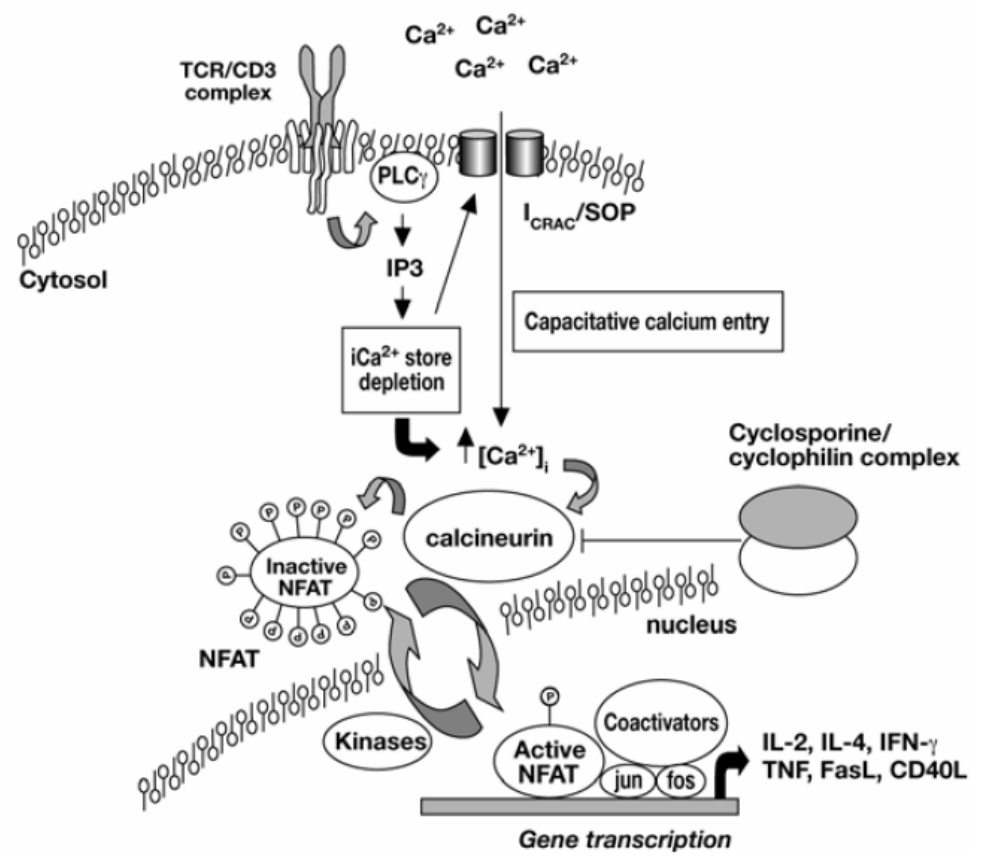
Escape medicatie

Voor deze groep patiënten zijn er geen farmacotherapeutische opties meer mogelijk, derhalve is escape medicatie niet van toepassing. Mochten er toch andere alternatieven mogelijk zijn, dan zullen die niet door de studie worden beperkt.

ONDERZOEKSGENEESMIDDEL

Naam en beschrijving

Ciclosporine A voor inhalatie. Ciclosporine A is een wit, volumineus poeder. Het poeder zit voorverpakt in een gesealde blister welke in een gesloten inhaler zit. Na openen van de blister via een treklipje is de inhaler gereed voor inhalatie.



Figuur 1. werkingsmechanisme ciclosporine A

Samenvatting van bevindingen uit niet-klinische studies

Farmacologie

Ciclosporine bindt aan ciclophylline en dit complex is een potente remmer van calcineurine, welke de expressie van een aantal cytokines reguleert waaronder interleukine-2 (Figuur 1). In aanwezigheid van ciclosporine worden transcriptie factoren van de NFAT (nuclear factor

activated T-cells) familie niet geactiveerd hetgeen resulteert in een falende activatie van interleukine-2, een belangrijke T-cel groei factor. De cellulaire respons is geinhibeerd op het moment van lymfocyt proliferatie. Daarom zou met lokale immuunsuppressieve therapie met ciclosporine de vroege intragraft immuunrespons kunnen worden gecontroleerd.

Farmacologische geneesmiddelinteracties
Er zijn geen specifieke studies verricht waarin geneesmiddelinteracties met geinhaleerd ciclosporine zijn onderzocht. Echter, het is te verwachten dat er lage systemische opname zal zijn van ciclosporine na inhalatie. Na verneveling van ciclosporine is dit ongeveer 5 tot 11% van de toegediende dosis. Na droog poeder inhalatie is dit naar verwachting ongeveer eenzelfde percentage. Geneesmiddelinteracties met ciclosporine na intraveneuze en orale toediening zijn wel goed omschreven en dat maakt het mogelijk om uitspraken te doen over mogelijke – systemische - interacties. Stoffen welke het CYP3A4 enzymsysteem beïnvloeden hebben een mogelijk effect op het metabolisme van ciclosporine. Daarnaast kunnen stoffen welke het CYP450 systeem induceren het metabolisme verhogen en de concentratie verlagen van ciclosporine.
Gebaseerd op de IB tekst van Neoral® is het volgende een samenvatting van geneesmiddelen welke een interactie met ciclosporine hebben. Geneesmiddelen welke de ciclosporine-spiegel kunnen verhogen zijn calciumblokkers (o.a. diltiazem, verapamil), antimycotica (o.a. fluconazol, itraconazol, ketoconazol), antibiotica (o.a. clarithromycine, erythromycine), glucocoricosteroiden (methylprednisolon), allopurinol, bromocryptine, danazol, metoclopramide, colchicine en amiodaron. Geneesmiddelen welke de ciclosporine-spiegel kunnen verlagen zijn antibiotica (nafcilline, rifampine), anticonvulsiva (carbamazepine, fenobarbital, fenytoine), octreotide en Sint Janskruid. Orlistat verlaagt de absorptie van ciclosporine in gezonde vrijwilligers met ongeveer 33%. Geneesmiddelen welke een interactie met ciclosporine hebben en tevens de renale disfunctie kunnen verergeren zijn NSAID's, aminoglycosiden, melfalan, amfotericine B, tacrolimus, cimetidine en ranitidine. Patiënten met psoriasis welke immuunsuppressieve stoffen of lichttherapie krijgen zouden niet tegelijkertijd ciclosporine mogen ontvangen omdat er een mogelijkheid is op een te sterk onderdrukt immuunsysteem.

Ciclosporine kan ook een effect hebben op de spiegels van andere geneesmiddelen. De methotrexaat-spiegel stijgt 30% en de spiegel van de 7-hydroxymetaboliet stijgt zelfs 80% bij gelijktijdig gebruik van ciclosporine. Er treedt een verlaagde klaring op van prednisolon, digoxine en lovastatine bij gebruik van ciclosporine. Ciclosporine mag niet worden gebruikt in combinatie met kaliumsparende diuretica omdat hyperkaliemie op kan treden. Vaccinatie kan verminderd effectief zijn bij gelijktijdig gebruik van ciclosporine en het gebruik van "levende" vaccins zou moeten worden vermeden.

Niet klinische farmacokinetiek en metabolisme

Met betrekking tot het onderzoeksgeneesmiddel van het hier voorliggende protocol zijn er geen dierstudies uitgevoerd. Wel zijn er dierstudies uitgevoerd met verneveld ciclosporine en deze gegevens worden hier gebruikt om informatie te verschaffen over ciclosporine na inhalatie. Deze dierstudies zijn uitgevoerd om te bepalen wat de werkzaamheid is van verneveld ciclosporine in long transplantatie modellen. Het bleek dat gezonde ratten na een eenmalige dosering van 3 mg/kg verneveld ciclosporine een 100-200x hogere longweefselconcentratie van ciclosporine hadden in vergelijking met lever, nier, hart en bloed. Na 24 uur was residueel ciclosporine alleen aanwezig in het long parenchym. Het oppervlak onder de curve ($AUC_{0-\infty}$) was ongeveer 46.000 ng·uur/gr in de long en 2.500 ng·uur/gr in bloed. Dit betekent dat de totale hoeveelheid in de long ongeveer een factor 20 hoger is dan in het bloed. In een model waarin de linkerlong was getransplanteerd in de rat gaf een dagelijkse dosis van 18 mg/kg een depositie van 3,6 mg/kg/dag.[15] Na 7 doses waren de ratten beschermd tegen allograft rejectie (gemiddelde graad 1,2±0,4; $p<0,05$) wat beter was dan intramusculair toegediend ciclosporine (gemiddelde graad 1,8±0,38). De schaal liep van 0, geen allograft rejectie, tot 4, vasculaire trombose and intra-alveolaire bloedingen. Als laatste is een studie in ratten uitgevoerd waarin onderzocht is in hoeverre verneveling of intramusculaire injectie van ciclosporine effectief was in het tegengaan van rejectie. [16] De resultaten toonden aan dat een systemische dosis van 15 mg/kg (intramusculair toegediend) ciclosporine een gelijkwaardige rejectie-score gaf als een depositie van 3 mg/kg ciclosporine (als aerosol toegediend).

Toxicologie

Ciclosporine

Systemische toediening: De systemische toxiciteit van ciclosporine is goed onderzocht in mens en dier. De farmacologische werkzaamheid en toxiciteit na systemische blootstelling in dieren zijn vergelijkbaar met de effecten in de mens.[17-20] De belangrijkste ongewenste effecten in de mens zijn, na toediening van klinisch relevante doseringen, renale disfunctie, hypertensie, dislipidemie, hirsutisme en tremor. Daarnaast bestaan een aantal minder vaak voorkomende bijwerkingen als hyperkaliemie en thrombocytopenie.

Pulmonale toediening: Er is een beperkte hoeveelheid gepubliceerde literatuur beschikbaar over de lokale toxiciteit (respiratoir systeem) van geïnhaleerd ciclosporine in dieren.[6] De primaire data over toxiciteit na pulmonale toediening is afkomstig uit twee belangrijke 28-daagse inhalatie-toxiciteit studies in ratten en honden. In deze studies werden de dieren maximaal tolereerbare (rat) of maximaal haalbare (hond) doses toegediend. Dit levert belangrijke informatie over de veiligheid van ciclosporine na inhalatie bij een maximale dosis en levert een schatting over de “worst case” toxiciteit.

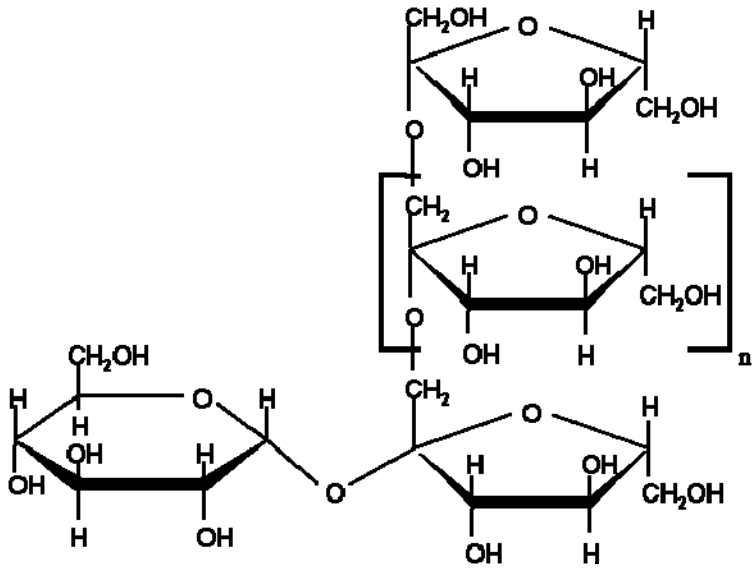
In de 28-daagse inhalatie studie in ratten kregen de dieren doses van 7,4, 24,3 en 53,8 mg/kg ciclosporine per inhalatie (verneveling) toegediend. Er waren behandeling-gerelateerde hematologische, serum en nier bevindingen welke overeenkomstig zijn met systemische blootstelling maar geen pulmonaire abnormaliteiten na histologische controle.

In de 28-daagse inhalatie studie in honden werden de dieren gedoseerd met 4,4, 7,6 en 9,6 mg/kg ciclosporine middels verneveling. Bij de hoge dosis was de respiratoire flowrate lager dan bij de andere groepen, hierdoor had de hoge dosis groep een geschatte vergelijkbare depositie als de groep met de middelste dosering. Dit toonde aan dat een langere doseertijd niet resulteerde in substantieel verhoogde toediening. Er waren geen ciclosporine-gerelateerde bijwerkingen. In honden werden geen klinisch relevante bijwerkingen in het respiratoire systeem gevonden.

Microscopisch onderzoek toonde in de groep met de middelste en hoogste dosering minimale en lokale ulceraties van het laryngeale slijmvlies aan, welke gepaard gingen met ontsteking. Deze ontstekingen waren

gemiddeld genomen ter grootte van een speldenpunt in een overig normaal uitziende larynx. Andere honden hadden geen laryngeale ontstekingen, hetgeen er op duidt dat de ontsteking niet duidelijk te relateren was aan de behandeling.

Inuline
Inuline is een natuurlijke grondstof die voorkomt in onder andere chigorij (witlof). Chemisch gezien bestaat inuline uit een glucose-eenheid die gepolymeriseerd is met fructose-eenheden (GF_n) (Figuur 2). Binnen de farmaceutische wereld is inuline nog nooit in inhalatie toegepast, daarom is er ook weinig informatie over de veiligheid van inuline. Inuline wordt daarentegen veel gebruikt in de voedselindustrie en wordt ook gebruikt als marker om de glomerulaire filtratiesnelheid te bepalen.[21-25]



Figuur 2. structuurformule inuline

Er is één artikel [26] welke de toxicologie van carboxymethylinulines (chemisch gemodificeerde inulines) beschrijft. In de betreffende studie is de sub-acute (4 weken) orale toxiciteit bepaalt in ratten tot een dosering van 1000 mg/kg/dag. Er werden geen behandelingsgerelateerde effecten gezien op lichaamsgewicht, voedselconsumptie, mortaliteit, hematologie, klinisch bloed beeld, gewicht van organen en pathologie. Verder veroorzaakte carboxymethylinuline geen dermale sensitisatie (als getest

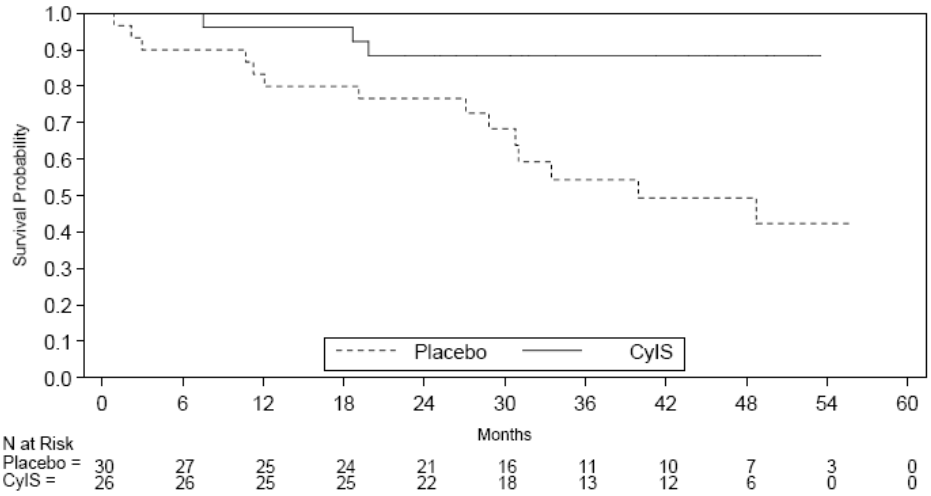
met cavia's) en mutagene activiteit (getest met Salmonella stammen en Escherichia coli). De resultaten met carboxymethylinuline zijn vergelijkbaar met andere natuurlijk voorkomende carbohydraten (sorbitol, sucrose, glucose) en oligofructoses. Derhalve is aannemelijk dat inuline geen toxiciteit in de gegeven dosering zal veroorzaken. De blootstelling aan inuline is in deze studie 12,5 mg/dag, wat overeenkomt met circa 0,18 mg/kg/dag voor een persoon van 70 kg. In §7.3.3.2 zal verder ingegaan worden op de veiligheid van inuline.

Samenvatting van bevindingen uit klinische studies
Samenvatting van uitkomsten uit klinische studies
In dit protocol is de studiemedicatie een poeder voor inhalatie. Met dit poeder zijn nog geen klinische studies uitgevoerd, maar wel met vloeistof voor verneveling welke ontwikkeld is door Chiron ontwikkeld als vloeistof voor verneveling. De laatste klinische studie heeft uiteindelijk geresulteerd in een New Drug Application (NDA 50-799) bij de FDA. De belangrijkste studie waarop NDA 50-799 is gebaseerd is een mono-center, gerandomiseerde en dubbel blinde klinische studie (ACS 001). Deze was ontworpen om de effectiviteit van geïnhaleerd ciclosporine op het verlagen van transplantaat-rejectie en verbeteren van de resultaten van long transplantatie. De klinische studie bevatte twee fasen: een initiele open label pilot fase en vervolgens een geblindeerde en gerandomiseerde fase. In de pilot fase ontvingen 10 patienten open label verneveld ciclosporine en werden vervolgens prospectief gevolgd om de veiligheid en tolereerbaarheid te onderzoeken. In de gerandomiseerde fase werden 56 patienten geincludeerd binnen 7 tot 42 dagen na enkele of dubbele long transplantatie en behandeld met ofwel verneveld ciclosporine of placebo. Van de 56 patienten ontvingen 26 patienten ciclosporine in propyleenglycol en 30 alleen propyleenglycol.

Alle patiënten ondergingen een initiele dosis-titratie periode om de maximaal verdraagbare dosis te vinden tot een maximum (protocollair vastgelegd) van 300 mg. Na deze 10-daagse periode dienden de patiënten de therapie drie keer per week te gebruiken voor een periode van twee jaar. Gedurende 24 tot 46 maanden vond follow-up plaats.

Van de placebogroep stierf 47 procent van de patiënten tegen 12% van de met ciclosporine-verneveling behandelde patiënten (Figuur 3). Dit

resultaat is een zowel klinisch als statistisch significante (log-rank P=0,007) verbetering. De niet-aangepaste schatting van het risico op dood onder de ciclosporine patiënten ten opzichte van placebo was 0,213 (95% betrouwbaarheidsinterval= 0,061; 0,743), hetgeen een reducite betekent van 79% op het overlijdensrisico als patiënten met verneveld ciclosporine worden behandeld. Het gevonden resultaat bleef men ook zien na stratificatie op transplantaattype, primaire diagnose, cytomegalovirus (CMV) positieve/negatieve donor match of mismatch of het optreden van acute afstoting (AR>2) voor de start van de studie medicatie. [6]



Figuur 3. Geschatte duur van overleving van long transplantaat; alle gerandomiseerde en behandelde patiënten

De werkzaamheid van verneveld ciclosporine wordt toegekend aan een verlaagde incidentie van chronische afstoting, in lijn met de lokale toediening van ciclosporine naar het luchtweg-epitheel. Patiënten welke behandeld waren met verneveld ciclosporine hadden een sterk verbeterde chronische afstoting vrije overleving in vergelijking met patiënten welke behandeld waren met placebo. Chronische afstoting werd gediagnosticeerd met ofwel een biopsie en onderzoek op obliterans bronchiolitis (histologisch onderzoek) ofwel klinisch op de aanwezigheid van het bronchiolitis obliterans syndroom (een onverklaarbare daling in FEV1 van 20% of meer). Binnen de placebogroep overleefde slechts 8% van de patiënten zonder chronische afstoting tegen 69% van de groep die

verneveld ciclosporine kreeg. Het risico op chronische rejectie of dood was 28% onder de ciclosporinegroep ten opzichte van de placebogroep (95% betrouwbaarheidsinterval=0,124; 0,630).

Klinische farmacokinetiek en metabolisme

Met het vernevelde en droog poeder product zijn geen distributiestudies uitgevoerd. Vanwege de toedieningsroute (inhalatie) kan echter wel worden verwacht dat in de longen de hoogste concentratie zal worden bereikt. Deze concentratie zal hoger zijn dan die verkregen met andere toedieningsroutes. Ciclosporine wordt wel geabsorbeerd via de long en gedistribueerd door het lichaam. Ciclosporine passeert de placenta en bevindt zich ook in moedermelk. Preferentiële opname van ciclosporine vindt plaats in de lever, pancreas en vetweefsel. Vanwege de lipofiliteit van ciclosporine wordt de bloed-hersen barriere nauwelijks gepasseerd. In plasma is de concentratie van ciclosporine omgekeerd evenredig met de hematocriet concentratie in het bloed. Ongeveer 33% tot 47% van het systemische ciclosporine wordt gevonden in plasma, 4% tot 9% in lymfocyten, 5% tot 12% in granulocyten en 41% tot 58% in erythrocyten. In plasma is ongeveer 90% gebonden aan lipoproteinen. Geneesmiddelen welke de binding van ciclosporine aan lipoproteinen beïnvloeden kunnen ook de klinische werkzaamheid beïnvloeden.
Uit Burckart et al [27] kan worden gevonden dat de tijd tot maximale concentratie na verneveling (Tmax) 0,68 (±0,30) uur is. De halfwaardetijd is 40,7 (±17,7) uur. De uitgebreide biotransformatie van ciclosporine leidt tot ongeveer 15 metabolieten. Biotransformatie vindt hoofdzakelijk plaats door middel van het cytochroom P450 afhankelijke mono-oxygenase systeem in de lever. De belangrijkste biotransformatie bestaat uit de mono- en dihydroxylering en N-demethylering van ciclosporine. Geneesmiddelen welke het CYP450 remmen of induceren kunnen de spiegels van ciclosporine verhogen of verlagen. De tot nu geidentificeerde metabolieten bevatten de intacte eiwitstructuur van de oorspronkelijke stof; sommige bezitten een zwakke immuunsuppressieve werking (tot een tiende van de onveranderde stof). De eliminatie kinetiek van ciclosporine is doorgaans variabel en gerelateerd aan het ziekteproces, de toestand van het getransplanteerde orgaan en de co-medicatie. Daarom is regelmatige controle van de bloedspiegels belangrijk.

De uitscheiding van ciclosporine vind hoofdzakelijk via de gal plaats, terwijl slechts 6% van een systemische dosis wordt uitgescheiden via de urine.

Veiligheid en effectiviteit in mensen

Veiligheid en effectiviteit ciclosporine

Uit de ACS001 studie van NDA 50-799 is gebleken dat ciclosporine een goed veiligheidsprofiel heeft. Lange termijn toedieningen leidde niet tot exacerbaties van bekende toxiciteit na orale of intraveneuze toediening. Specifiek gezien was er geen bewijs voor een verhoogd risico op nefrotoxiciteit, neurotoxiciteit, een verhoogde kans op infecties, of een verhoogd risico op maligniteiten. De vernevelvloeistof werd wel geassocieerd met irritatie van de longen en bronchospasmen, maar de bijwerkingen waren over het algemeen mild tot matig van aard en begonnen vroeg na de start van de therapie. De bijwerkingen leidden niet tot ernstige respiratoire complicaties.

Omdat het onderzoeksgeneesmiddel in dit protocol geen propyleenglycol bevat zoals het product beschreven in NDA 50-799 zullen er vooral uitspraken worden gedaan over de veiligheid van ciclosporine per inhalatie. Overigens is van propyleenglycol gerapporteerd dat het irritatie en bloedingen kan veroorzaken in honden en ratten (zie 2^{e} alinea, pagina 7 van [7], bijgevoegd als bijlage). Omdat inuline nog niet is toegepast in inhalatie zal hier nog nader op in worden gegaan.

De data over veiligheid van ciclosporine per inhalatie is afkomstig uit twee studies: ACS001 en ACS002 (NDA 50-799). In studie ACS001 waren 66 patienten geincludeerd en in ACS002 (een safety studie) waren 70 patienten geincludeerd in 1 van de 7 protocol-armen.

Vanuit ACS001 rapporteerden alle patiënten één of meer AE's en AE's werden ingedeeld in 24 van de 26 MedDRA System Organ Classes (SOCs). De meeste SOCs bevatten meer dan 35% van de patiënten (zie tabel 1).

Het grootste aantal gemelde bijwerkingen was afkomstig uit de Respiratoir/Thorax/mediastinum classificatie. Van de placebogroep gaf 97% van de patiënten aan hier bijwerkingen te ervaren (dit moet afkomstig zijn van propyleenglycol) tegen 100% van de ciclosporinegroep. Het bleek dat er een syndroom van bronchiale irritatie en luchtwegobstructie optrad in de ciclosporinegroep. De groep welke ciclosporine per verneveling kreeg had een hogere incidentie van

bronchospasmen, hoest, geexacerbeerde dyspnoe, excessieve bronchiale secretie, luchtweginfecties en als irritant ervaren piepen. Dit was waarschijnlijk een direct effect van ciclosporine in de luchtweg en was niet verwacht gezien de route van toediening. De overgrote meerderheid van de patiënten merkte op dat dit type bijwerkingen vooral optrad bij aanvang van de medicatie, tijdens de dosis-escalatie fase. De meeste patiënten hadden aanvakelijk premedicatie als bronchodilatoren en lokale anesthesie nodig, maar het gebruik hiervan werd verminderd na 10 dagen therapie. Bronchiale reactiviteit lijkt de belangrijkste bijwerking te zijn van geïnhaleerd ciclosporine.

Tabel 1. Bijwerkingen na verneveling van ciclosporine

MedDRA System Organ Class	Adverse Event	Placebo (n=30)	Ciclosporine-verneveling (n=26)
Oog	Wazig zien	8 (27%)	15 (42%)
Gastrointestinaal	Overgeven	0	4 (11%)
Plaats van toediening	Borstpijn	6 (20%)	15 (42%)
Onderzoek	hartruis	0	6 (17%)
Metabolisme/voeding	Hyperlipidemie	0	5 (14%)
Musculatuur/bind-weefsel/bot	Rugpijn	3 (10%)	12 (33%)
Centraal zenuwstelsel	Slaperigheid	0	6 (17%)
Psychiatrie	Verwardheid	0	4 (11%)
Renaal/urinaal	Alle	19 (63%)	29 (81%)
	Frequentie van urineren	5 (17%)	14 (39%)
	Polyurie	1 (3%)	6 (17%)
Voortplantingssysteem	Alle	4 (13%)	10 (28%)
Respiratoir/thorax/ mediastinum	Acuut respiratoir falen	0	4 (11%)
	Hoest	8 (27%)	18 (50%)
	Geexcacerbeerde dyspnoe	4 (13%)	11 (31%)
	Pharyngitis	5 (17%)	14 (39%)
	Respiratoire stoornis	0	7 (19%)
	Ademhalingskanaal stoornis	1 (3%)	6 (17%)

Uit ACS002 is gebleken dat de SAE's niet het gevolg zijn van inhalatie van ciclosporine. De gerapporteerde bijwerkingen in deze studie waren vergelijkbaar met die in ACS001.
De reden waarom patiënten de studie niet af konden maken was het gevolg van kortademigheid, irritatie van de luchtwegen en obstructie van de luchtwegen.

Veiligheid inuline
Veruit de meeste informatie over inuline is bekend uit de voedselindustrie. Meestal betreft het dan gegevens over orale toediening. Inuline wordt niet opgenomen in het maagdarmkanaal (niet verteerbaar) en wordt veel gebruikt als vet en suiker vervanger voor diverse producten. Als zodanig wordt de dagelijkse inname van inuline geschat (in Europa en de Verenigde Staten) op 3 tot 11 gram. Dit feit is de hoeksteen van de veiligheidsoverwegingen van inuline. Voorzover bij ons bekend zijn er in de literatuur geen specifieke aanwijzingen om de veiligheid van inuline te betwijfelen. Hoewel dit geen bewijs is van veiligheid is het wel een comfortabel feit.[21-23, 28, 29]
Uiteraard wordt inuline in de voedselindustrie alleen oraal ingenomen. Het feit dat het een niet verteerbaar molecuul is maakt dat het systemisch gezien niet wordt opgenomen. Na orale inname wordt het via de faeces weer uitgescheiden. Dit is uiteraard anders na bijvoorbeeld inhalatie of injectie. Het verloop van inuline is dan anders. Inuline wordt al sinds 1931 gegeven als intraveneuze injectie om de glomerulaire filtratiesnelheid (GFR) te bepalen. Dit is een standaard procedure geworden zonder enige toxiciteitseffecten.[24, 25] Tot op heden is deze methode de referentiemethode wanneer andere procedures getest worden om de GFR te meten.[24] Inuline is een ideale stof om de GFR te bepalen omdat inuline vrijelijk wordt gefilterd door de nieren van de bloedbaan, niet wordt gemetaboliseerd en geen tubulaire secretie of absorptie ondergaat. Met andere woorden, wanneer inuline in de bloedbaan aanwezig is wordt het gefilterd door de nieren en onveranderd uitgescheiden in de urine. Na inhalatie zal een deel worden opgenomen in de bloedbaan (net als ciclosporine) en een deel zal worden geklaard via het maagdarmkanaal. In het eerste geval zal inuline volledig oplossen en geklaard worden via de longmembraan. Daarna vindt filtering in de nier plaats en wordt inuline via de unrine onveranderd uitgescheiden. In het tweede geval zal een deel direct van de formulering direct worden

ingeslikt (dat gedeelte wat niet in de long terechtkomt) en een deel zou via de mucociliaire lift naar het maagdarmkanaal worden gebracht. In dit geval zal inuline onveranderd via de faeces worden uitgescheiden. Er zal in ieder geval geen accumulatie plaatsvinden van inuline in het lichaam dankzij de goede wateroplosbaarheid.
In 1992 heeft een commissie van experts middels een review van tot dan gepubliceerde studies dan ook geconcludeerd dat “er geen reden is om te geloven dat oligofructoses of hun metabolieten potentieel toxisch zijn door vergroot gebruik in voedsel; in tegendeel, recente bevindingen beschrijven de gezondheidsbevorderende voedingseigenschappen van deze stoffen”. In 2002 heeft de FDA een Generally Regarded as Safe (GRAS) statement gegeven over inuline.[30]
Bij de vakgroep farmaceutische technologie en biofarmacie is wel eenmalig inuline via inhalatie toegediend aan muizen om te onderzoeken in hoeverre dit effect had op de pathologie van longweefsel; dit bleek niet verschillend te zijn van muizen die niets kregen.[Data on file]
Gezien de gegevens die tot op heden bekend zijn over inuline wordt het gelegitimeerd geacht om inuline te gebruiken als farmaceutische hulpstof. Uiteraard is bekend dat het gebruik van een nooit eerder gebruikte hulpstof een bepaald risico met zich meedraagt, maar dat risico is volgens de onderzoekers verwaarloosbaar.

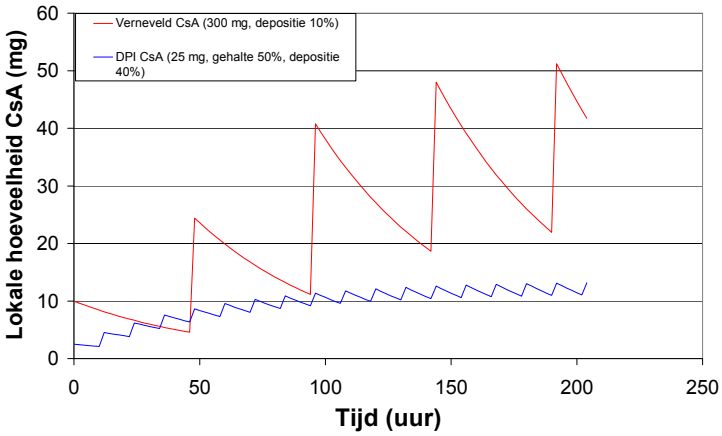
Samenvatting bekende en mogelijke risico’s en voordelen
Hoewel alle data tot nu toe is gegenereerd uit verneveling van ciclosporine in één centrum, is gebleken dat inhalatie van ciclosporine effectief is in het verlagen van mortaliteit door het verminderen van de incidentie van chronische rejectie. Dit is ook gebleken uit andere studies.[4, 5]
De risico’s van inhalatie van ciclosporine zijn klein. Zo is de systemische concentratie na verneveling van ciclosporine minder dan 15% van de systemische concentratie na orale toediening. Een vergelijkbare ratio is te verwachten na droog poeder inhalatie. Deze lage systemische concentratie geeft geen aanleiding tot extra systemische toxiciteit als nefrotoxiciteit, neurotoxiciteit en maligniteiten.
De bijwerkingen van ciclosporine na verneveling (vergelijkbaar met droog poeder inhalatie) waren hoofdzakelijk pulmonaal gerelateerd. Het meest genoemd werden hoest, piepen, en dyspnoe en waren hoogstwaarschijnlijk gerelateerd aan bronchospasmen.

Ciclosporine is een standaardbehandeling voor meer dan 20 jaar. Het veiligheidsprofiel van ciclosporine is goed bekend en het is daarom onwaarschijnlijk dat er nog nieuwe systemische toxiciteiten gevonden zullen worden.

Beschrijving en rechtvaardiging toedieningsroute en dosering
Als route voor toediening is gekozen voor inhalatie. Vanwege de ernstige toxiciteit in de klinisch gehanteerde orale doses na longtransplantatie is het verlagen van de systemische dosis prioriteit één. Dit leidt vaak tot dosisaanpassingen op geleide van het klinische beeld (graad van afstoting en nierfunctie). Met een slechte nierfunctie is de dosis dermate laag dat het risico op chronische afstoting drastisch toeneemt. Door ciclosporine per inhalatie aan te bieden kan de systemische dosis laag worden gehouden en wordt een situatie bereikt waarin optimale bescherming tegen chronische afstoting met weinig systemische toxiciteit hand in hand gaan. Dit verlaagt de mortaliteit en morbiditeit.
Uit literatuur is gebleken dat meer dan 5 mg ciclosporine in de longen effectief is in het verminderen van chronische afstoting. Het wordt geschat dat de depositie na inhalatie van het poeder ongeveer 40% is, dat wil zeggen dat 40% van de aangeboden dosis op de plaats van werking komt. Daarnaast is het gehalte aan ciclosporine in de formulering 50%. Met deze gegevens kan worden teruggerekend wat de dosering dient te zijn:
De dosis is (100%/40%)*(100%/50%)*5 mg= 25 mg van een 50% ciclosporine/50% inuline formulering. Dit wordt gegeven in twee of drie doses van ieder 12,5 mg poeder.

De getallen en schattingen zijn als volgt verkregen. Uit de literatuur (verneveling) blijkt dat minimaal 5 mg CsA nodig is in de longen om effectiviteit te halen. Uit eerder onderzoek over droog poeder inhalatie van colistine[31, 32] is gebleken dat droog poeder inhalatie met behulp van een op een air classifier gebasseerde inhaler zeer effectief is. In deze studie wordt eenzelfde soort inhaler gebruikt. Het is gebleken dat 25 mg droog poeder inhalatie effectiever was dan verneveling van 160 mg colistine wanneer gebruik werd gemaakt van een inhaler met een air classifier. Er werd bij droog poeder inhalatie een hogere serum concentratie gemeten en Tmax was eerder bereikt bij droog poeder inhalatie in vergelijking met verneveling. Mede door deze data wordt de

depositie van ciclosporine A als droog poeder geschat op 40%. Daarnaast is de farmacokinetiek van ciclosporine na verneveling en droog poeder uitgerekend op basis van in de literatuur gevonden waarden en schattingen. Voor verneveling zijn alle benodigde gegevens bekend: dosis (300 mg), dosisopbouw (1^{e} dosis is 100 mg, 2de dosis is 200 mg, 3de en volgende doses zijn 300 mg), doseerfrequentie (eens in de twee dagen), depositie (10%) en halfwaardetijd (40,7 uur). Voor droog poeder inhalatie zijn de volgende getallen gebruikt:



Figuur 4. Berekende en geschatte farmacokinetiek van ciclosporine als verneveling en droog poeder inhalatie

Dosis: 12,5 mg per inhalatie
Frequentie: 2x per dag
Gehalte CsA in formulering: 50%
Depositie: 40%
Uit Figuur 4 blijkt dat de hoeveelheid ciclosporine opbouwt in de loop van de tijd, na circa 100 uur is een stabiele spiegel bereikt van ca. 10 mg. Dit ligt boven de beschreven spiegel van 5 mg en wordt dus als voldoende geacht. Indien de depositie van droog poeder inhalatie lager blijkt te zijn, dan dient na heroverweging de dosis op geleidde van de depositiestudie aangepast te worden.

Doseringen, dosisaanpassingen en methode van toediening
Ciclosporine-poeder voor inhalatie wordt dagelijks in twee of drie doses gegeven van 12,5 mg elk. Iedere dosis bevat 6,25 mg ciclosporine en

6,25 mg inuline. Ciclosporine wordt via inhalatie, als droog poeder, te worden toegediend.
Routinematig zal de ciclosporine-spiegel in patiënten worden gemeten. Daarnaast wordt er bij de start van de studie een depositiestudie uitgevoerd. Van alle geincludeerde patienten wordt de depositie bepaald. Op basis van het gemiddelde wordt het aantal doses bepaald. Alle patienten worden met dezelfde dosis behandeld (dwz 2 of 3 doses per dag). Is de depositie gemiddeld lager dan 40% dan worden drie doses gegeven en is de depositie hoger dan 40% dan wordt voor twee doses gekozen.
Dosisaanpassingen worden gegeven per inhaler (12,5 mg poeder). Een te lage dosis is het minst wenselijk, een hoge dosis is minder gevaarlijk gezien de berekende farmacokinetiek (Fig 4). Ten aanzien van de hoeveelheid doses kan worden gemeld dat twee doses met een depositie van 40% dezelfde berekende piekspiegels geeft als drie doses met een depositie van 30%.

Productie en etikettering van onderzoeksgeneesmiddel
Het geneesmiddel wordt onder verantwoordelijkheid van de ziekenhuisapotheek geproduceerd onder de geldende kwaliteitsnormen (GMPz). De betreffende productieruimtes zijn vrij gegeven voor productie en er wordt gewerkt met productieprotocollen. Voordat tot productie is overgegaan, wordt het product onderworpen aan ontwerpkwaliteit waarin zaken als grondstoffen, farmacologie, werkzaamheid en dergelijke aan bod komen. Hiertoe is een dossier aangelegd.

Drug accountability
Voor de logistiek van het onderzoeksgeneesmiddel draagt de ziekenhuisapotheek van het UMCG zorg. Per patient wordt na het eerste bezoek voor twee weken aan geneesmiddel meegegeven. Na het tweede bezoek is dat steeds voor 4 weken. Bij ieder bezoek dient de patient de gebruikte inhalatoren te retourneren aan de behandelend arts die ze vervolgens aan de ziekenhuisapotheek doet toekomen. Door telling van uitgegeven en geretourneerde inhalatoren kan per patient het geneesmiddelgebruik worden gevolgd.

RADIOFARMACON VOOR DEPOSITIE

Voor het vaststellen van de dosis is het van belang om te weten welk percentage van het geneesmiddel in de longen komt (zie ook §9.1.3.1; depositie en §3.3 Andere doelstellingen). Er is uitgegaan van een theoretisch percentage van 40%; met dat percentage is een dosis geschat van twee maal daags 12,5 mg (zie ook §7.5). Het doel van de depositiestudie is drieledig:

1) De patienten krijgen een inhalatie-instructie en feedback over hun inhalatie-manoeuvre.
2) De onderzoekers krijgen inzicht over de depositie van het onderzoeksgeneesmiddel en efficientie van de inhaler wat kan leidden tot eventuele verbeteringen.
3) De depositiestudie kan aanleiding geven tot dosisaanpassingen zodat onder- en overdosering kan worden voorkomen.

Het onderzoeksgeneesmiddel wat gebruikt wordt voor het meten van de depositie is de bestaande formulering waarin ciclosporine A vervangen is door Amerscan Hepatate II Agent ([^{99m}Tc]-technetium-tincolloid), een geregistreerd middel. ^{99m}Technetium/tincolloid wordt geproduceerd door radiofarmacie, afdeling Nucleaire Geneeskunde in het UMCG onder verantwoordelijkheid van de ziekenhuisapotheek. Bereiding van het poeder voor inhalatie is identiek aan bereiding poeder met ciclosporine alleen dan met radioactiviteit. In verband met het gebruik van radioactiviteit zal deze bereiding plaatsvinden in het B/lab van de radiofarmacie op de afdeling Nucleaire Geneeskunde. De dosis die de patienten binnen zullen krijgen is ongeveer 50 MBq. Het middel dient te voldoen aan de vigerende eisen als gesteld in de Europese Pharmacopee en wordt dan ook als zodanig getest. De stralingsbelasting van de patient is geschat op 1,7 mSv na een dosering van 50 MBq. De schatting voor de stralingsbelasting is als volgt tot stand gekomen. Bij inhalatie van het ^{99m}Tc-gelabelde micro-sferen zal 90% in de long worden opgenomen, 10% blijft in de inhalator. Bovendien zal er geen overdracht naar het bloed plaatsvinden. Voor de stralingsbelasting van een dergelijk ligand kan het Effective Dose Equivalent (EDE, ICRP 53 p. 10) op grond van het farmacologisch gedrag worden uitgerekend. Aangenomen wordt dat 50% van de gamma energie in het longweefsel wordt gedeponeerd. Dit betekent dat er per toegediende MBq No= 2.805E+10 kernen zullen vervallen in het longweefsel. De stralingsbelasting wordt dan 2.8-04

J/MBq in de long. Bij een gewicht van 1 kg (MIRD) wordt de stralingsbelasting van de long 0.28 mSv/MBq. Voor de bijdrage tot het EDE moet gerekend worden met een gewichtsfactor van 0.12 (ICRP 53). Daar er geen verdere verspreiding door het lichaam plaatsvindt is de EDE voor ^{99m}Tc-microsferen 0.034 mSv/MBq. Bij een dosering van 50 MBq wordt de stralingsbelasting 1.7 mSv (EDE).

Het meten van de depositie gebeurt na inhalatie van het radioactieve onderzoeksgeneesmiddel (zonder ciclosporine) via de inhaler die ook gebruikt wordt in de rest van de studie. Enkele minuten na inhalatie wordt door de afdeling Nucleaire Geneeskunde anterior en posterior een statische scan opgenomen. Ook de inhalaer wordt gemeten. De scan wordt vervolgens uitgewerkt door de afdeling Nucleaire Geneeskunde (mond/keel holte, long perifeer en centraal en inhaler) om een massabalans te kunnen bepalen van het radioactief gelabelde poeder.

Het bepalen van depositie van droog poeder inhalatie met technetium is iets wat al geruime tijd gebeurt.[33-35] Het is een geruststellend feit dat vaker studies zijn uitgevoerd met technetium in het kader van depositie van droog poeder inhalatie. In het hier gebruikte proces wordt technetium ingesloten in het deeltje voor inhalatie en is daarom uitermate geschikt voor het uitvoeren van de depositie bepaling omdat technetium terecht zal komen daar waar ook het geneesmiddel terecht komt. Gezien de verwachting met het onderzoeksgeneesmiddel zal er ook in het geval van het radiofarmacon niets achterblijven in de long en wordt alles geklaard uit het lichaam.

METHODEN

Studie parameters/eindpunten

Primaire studie parameter/eindpunt

De primaire studieparameter is de “Forced Expiratory Volume in the first second” (FEV_1). Deze waarde vormt de basis voor de stadiëring van het bronchiolitis obliterans syndroom, zie tabel 2.[36]
In deze studie worden patiënten met ongestoorde transplantaatfunctie (BOS 0 stadium) niet blootgesteld aan het onderzoeksgeneesmiddel. De reden hiervoor is dat ciclosporine als rescue therapie wordt ingezet, dat wil zeggen bij die patienten waar geen farmacotherapeutische opties meer voorhanden zijn. Uit de literatuur is bekend dat na vernevelen van

ciclosporine (300 mg CsA in propyleen glycol) de afname van de FEV_1 in de loop van de tijd (chronische afstoting is een progressief verslechterende aandoening) kan worden geremd.[38, 39] In deze studie willen we hetzelfde voor de droog poeder inhalatie proberen te bewerkstelligen. Indien de afname in FEV_1 kan worden geremd ten opzichte van het FEV_1 beloop voorafgaand aan de interventie is droog poeder inhalatie van ciclosporine effectief. Deze internationaal erkende benadering [36] is aangegeven in de onderstaande figuur 5.

Tabel 2. Stadium BOS en correlatie met longfunctie

BOS stadia	Longfunctie
BOS 0	FEV_1>90% t.o.v. basislijn en FEF_{25-75}>75% t.o.v. basislijn
BOS 0-p	FEV_1 81%-90% t.o.v. basislijn en/of FEF_{25-75}≤75% t.o.v. basislijn
BOS 1	FEV_1 66%-80% t.o.v. basislijn
BOS 2	FEV_1 51%-65% t.o.v. basislijn
BOS 3	FEV_1 <50% t.o.v. basislijn

Secundaire studie parameter/eindpunt
Inhalatie van ciclosporine bovenop de bestaande therapie mag geen extra nefrotoxiciteit mag veroorzaken. Nierfunctie wordt standaard bij elke poliklinische controle gemeten (serumkreatinine) en bovendien met behulp van de glomerulaire filtratie snelheid (GFR) voorafgaand aan en bij het einde van de interventie. Hiertoe worden de patiënten actief gevolgd. Deze parameter wordt meegenomen omdat het uiteindelijke doel voor droog poeder inhalatie van ciclosporine het voorkomen/behandelen van chronische afstoting na longtransplantie én het voorkomen van ernstige systemische bijwerkingen (neuro-/nefrotoxiciteit) is.

Andere studie parameters
Depositie
Van belang is om te weten hoeveel ciclosporine daadwerkelijk in de longen komt. Hiertoe zal van iedere patient worden gevraagd om radio-actief gelabeld inuline (dwz. zonder de werkzame stof, ciclosporine) te inhaleren. Hiermee zal inzicht worden verkregen in de echte depositiegegevens en zal het mogelijk zijn effecten te correleren aan de dosis. Zie §8 voor een uitgebreide uitleg over het radiofarmacon voor depositie.

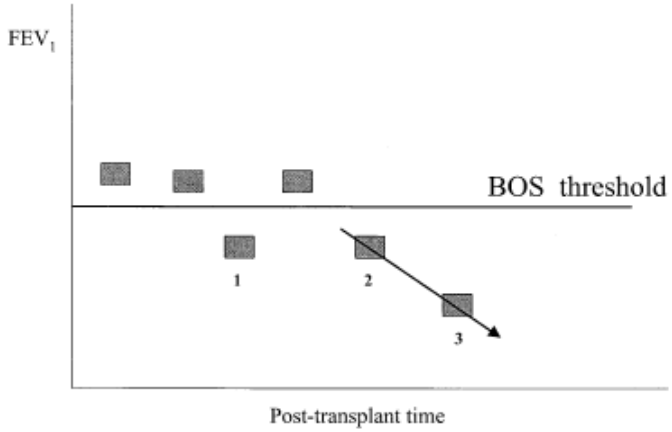


FIGURE 1 Event 1: drop below BOS threshold, not validated by second measurement. Event 2: first BOS measurement and time of onset of BOS defined by validating event #3. FEV_1 decline = slope of values 2 and 3 and any additional measurement over a 1–3 month period.

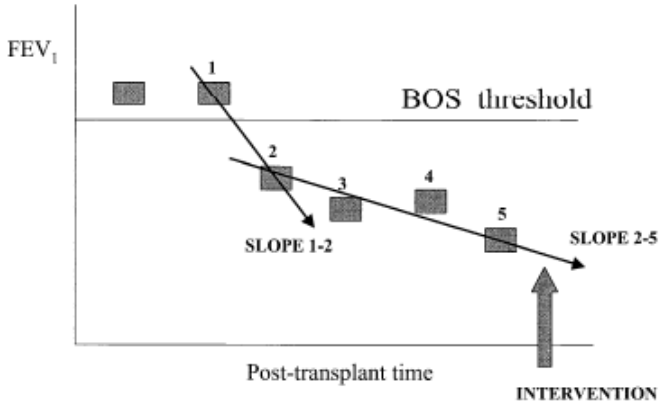


FIGURE 2 Though initial decline below BOS threshold shows a steep decline (slope 1–2), preintervention value 2 which defines BOS onset (and is validated by subsequent values) and subsequent values 3–5 define the slope prior to intervention. Benefit of therapeutic intervention will be defined by comparison with the slope 2–5.

Figuur 5.

Bloedspiegel
Van de ciclosporine dosis die lokaal in de longen terechtkomt zal een deel worden geabsorbeerd in de systemische circulatie. Uit de literatuur is gebleken dat dit circa 10% van de oorspronkelijke dosis is. Bij ieder bezoek wordt de bloedspiegel gecontroleerd om de veiligheid van ciclosporine bij te houden in verband met de mogelijke nephro-/neurotoxiciteit.

Randomisatie/blindering
Binnen deze studie zal er geen randomisatie plaatsvinden vanwege de gekozen studie opzet. De studie opzet is gekozen vanwege het feit dat er geen farmacotherapeutische opties meer voorhanden zijn voor deze patiënten. Als resultante van het behandelingsprincipe vindt er ook geen blindering plaats.

Studie procedures
In tabel 3 staan de bepalingen weergegeven welke worden voor deze studie nodig zijn.

Tabel 3. studieprocedures

	Procedure	Standaard -protocol	Extra voor studie	Invasief (J/N)	Tbv. Studie-parameter
Depositie	Inhalatie radio-actief materiaal		√	J	Ander
Medicatie	Standaardmedicatie	√		N	
	Studiemedicatie		√	J	
Bepalingen	Longfunctie	√		N	Primair
	Nierfunctie	√		J	Secundair
	Bloedspiegel-bepaling*	√	√	J	Ander
Medische controle	Routine controle	√		N	

* Bij de bloedspiegel wordt naast de standaard bloedwaarde bepalingen extra op ciclosporine gecontroleerd.

Tabel 4. visites

Bezoek	Tijd (weken)	Procedures
Routine controle		Voorstel studieparticipatie/inclusie-gesprek
0		Informed consent/Uitleg studie
1	0	Uitleg inhalatie procedure Uitvoeren depositiestudie Routine controle (waarin o.a. longfunctie, nierfunctie) GFR Afleveren studiemedicatie (3 wkn)
2	2	Routine controle Bepalen ciclosporine-bloedspiegel (dalspiegel en extra alleen dit bezoek twee uur na inhalatie) Afleveren studiemedicatie (3 wkn) Innemen gebruikte inhalatoren
3	4	Routine controle Bepalen ciclosporine-bloedspiegel Afleveren studiemedicatie (4 wkn) Innemen gebruikte inhalatoren
4	8	Routine controle Bepalen ciclosporine-bloedspiegel Afleveren studiemedicatie (4 wkn) Innemen gebruikte inhalatoren
5	12	Routine controle Bepalen ciclosporine-bloedspiegel Afleveren studiemedicatie (4 wkn) Innemen gebruikte inhalatoren
6	16	Routine controle Bepalen ciclosporine-bloedspiegel Afleveren studiemedicatie (4 wkn) Innemen gebruikte inhalatoren
7	20	Routine controle Bepalen ciclosporine-bloedspiegel Afleveren studiemedicatie (4 wkn) Innemen gebruikte inhalatoren
8	24	Routine controle Bepalen ciclosporine-bloedspiegel GFR Afleveren studiemedicatie (4 wkn) Innemen gebruikte inhalatoren

Voor de studie zijn weinig extra procedures nodig. Alleen het geven van speciale medicatie (voor de depositiestudie en studiemedicatie) is een extra belasting voor het ziekenhuis en patient. Daarnaast dient het laboratorium een aantal extra bepalingen uit te voeren op de spiegel van ciclosporine.
De totale studieduur bedraagt 6 maanden, hierin dienen de patiënten een aantal keer in het ziekenhuis langs te komen. Zie tabel 4 voor het aantal visites en de te volgen procedures.

Terugtrekken
Patiënten mogen zich op ieder tijdstip terugtrekken uit de studie zonder persoonlijke gevolgen. Indien nodig kan de behandelend arts ervoor kiezen om een patient uit de studie te zetten. Dit kan gebeuren op geleide van het klinische beeld (zie §9.7 tabel 5), maar ook bij het niet kunnen volgen van het studieprotocol.

Specifieke criteria voor terugtrekken
Er zijn geen specifieke criteria vastgesteld.

Follow-up van teruggetrokken patient
Indien een patient zich terugtrekt of uit de studie wordt gezet zal deze patient vervallen in het originele follow-up systeem van de longtransplantatie eenheid. Indien er sprake is van een niet-verwachte verslechtering van het klinische beeld door de studiemedicatie zullen er adequate maatregelen worden getroffen op aanraden van de behandelend arts.

Voortijdige beëindiging van studie
De studie zal voortijdig worden beëindigd indien onverhoopt blijkt dat het onderzoeksgeneesmiddel dusdanig onveilig is en de patiënten een groot risico lopen dat het niet ethisch wordt geacht om de studie voort te zetten.
In deze studie zijn de criteria voor voortijdige beëindiging van de studie gedefinieerd op geleide van de veiligheidsgegevens van verneveld ciclosporine en op geleide van de relatieve ernst van de bijwerkingen.
Voor iedere patient wordt individueel door de behandelend arts bepaald of het verantwoord is om de studiemedicatie voort te zetten indien deze één van de aan de studiemedicatie gelieerde bijwerkingen heeft uit

tabel 5. De genoemde bijwerkingen hebben direct invloed op de longfunctie en wegen daarom zwaar. Dit betekent overigens niet dat andere bijwerkingen uitgesloten worden in de beschouwing.
Indien meer dan 40% van de patiënten met dezelfde bijwerking als genoemd in tabel 5 met de studie stopt of uit de studie wordt gehaald zal de Data Safety Monitoring Board bijeen komen en bespreken of de betreffende bijwerking een groepseffect betreft of dat deze individueel wordt bepaald. De uitkomst van dit gesprek zal bepalen of de studie voortijdig wordt gestopt of niet.

Tabel 5. bijwerkingen welke aanleiding geven tot discussie op doorgaan studie en medicatie

Hoest	Acuut respiratoir falen
Geëxacerbeerde dyspnoe	Pharyngitis
Respiratoire stoornis	Ademhalingsstoornis
Chronische luchtweginfectie	

VEILIGHEIDSRAPPORTEN

Sectie 10 WMO

Volgens artikel 10, eerste lid van de Wet medisch-wetenschappelijk onderzoek met mensen zal de uitvoerder van het onderzoek het aan de toetsende commissie melden als het onderzoek '*…een verloop neemt dat in noemenswaardige mate ongunstiger is voor de proefpersoon dan in het protocol is voorzien…*'. De uitvoering van het onderzoek wordt opgeschort totdat een nader oordeel is gegeven (tenzij de gezondheid van de proefpersoon opschorting niet toelaat).

Ongewenste voorvallen en ernstige ongewenste voorvallen

Het GCP richtsnoer definieert een ernstig ongewenst voorval (SAE) of ernstige bijwerking (Serious ADR) als: *elk ongewenst medisch voorval dat bij ongeacht welke dosis:*

- *de dood tot gevolg heeft,*
- *levensbedreigend is,*
- *ziekenhuisopname of verlenging van opname noodzakelijk maakt,*
- *tot blijvende of ernstige invaliditeit/arbeidsongeschiktheid leidt, of*
- *een aangeboren afwijking/geboorteafwijking is. (artikel 1.50 GCP)*

Al deze SAE's zullen aan de METc worden gemeld volgens de procedures van de METc.
Indien er een SAE optreedt waarbij er een verwachte (waarschijnlijk of zeker) relatie is tot de onderzoeksmedicatie zal dit worden gemeld aan de registratie-autoriteiten. In het geval van de onderzoeksmedicatie in deze studie is gesteld dat deze niet in Nederland is geregistreerd. Derhalve is het relevante orgaan de Hoofdinspectie voor de Farmacie en de Medische Technologie, Inspectie voor de Gezondheidszorg.

Verdachte en onverwachte ongewenste voorvallen
Verdachte en onverwachte ongewenste voorvallen zijn ongewenste voorvallen welke van nature of in ernst niet consistent zijn met de huidige product informatie (Investigator's Brochure).
De onderzoekers zullen alle verdachte en onverwachte ongewenste voorvallen melden aan het College ter Beoordeling van Geneesmiddelen en geaccrediteerde METc. Het rapporteren zal niet langer dan 15 dagen duren nadat de onderzoeker van het voorval heeft kennis genomen. Voor fatale of levensbedreigende voorvallen wordt binnen maximaal 7 dagen een voorlopig rapport opgesteld en wordt maximaal 8 dagen genomen om tot een definitief rapport te komen.

Jaarlijks veiligheidsrapport
De studie heeft een duur van 6 maanden. Daarom zal er niet een jaarlijks veiligheidsrapport worden opgesteld.

Follow-up van ongewenste voorvallen
Alle ongewenste voorvallen worden gevolgd tot ze zijn afgenomen of tot er een klinisch stabiele situatie is ontstaan. Afhankelijk van het voorval zijn mogelijk additionele testen of medische procedures nodig en/of verwijzing naar een huisarts of medisch specialist.

Data Safety Monitoring Board (DSMB)
Voor deze relatief kleine studie bestaat de Data Safety Monitoring Board uit de projectleider (Prof. Dr. H.W. Frijlink), de hoofdonderzoekers (Dr. W. van der Bij en Drs. G.S. Zijlstra) en de onafhankelijk arts (Prof. Dr. H.A.M. Kerstjens) vanwege het feit dat deze personen de studie van dichtbij volgen en daarom snel inzage hebben in eventuele veiligheidsproblemen. Daarnaast is de samenstelling van de monitoring

board divers met expertise van apothekers, een arts gespecialiseerd in het behandelen van longtransplantatiepatiënten en een longarts.

STATISTISCHE ANALYSE

Beschrijvende statistiek

De gemeten waarden zullen als discrete waarden worden genomen. De waarden van de pre-interventie controles (afname FEV_1, GFR) worden gemiddeld evenals de waarden van de interventie (afname FEV_1, GFR). Ook andere gegevens als leeftijd, type transplantatie en primaire diagnoese worden meegenomen. Per patient worden de verkregen gegevens in een grafiek uitgezet voor wat betreft de longfunctie voor en tijde van de studie. Dit wordt ook gedaan met de ciclosporine bloedspiegels, serumcreatininewaarden en GFR voor en na de studie.

Univariate analyse

De waarden voor afname in FEV_1 pre-interventie en post-interventie zullen middels een gepaarde t-toets met elkaar vergeleken worden op significantie. Ook wordt de GFR pre- en post-interventie met een gepaarde t-toets vergeleken. Uiteraard is de steekproef te klein om een uitspraak te doen over een eventueel groepseffect. De resultaten worden als richting-gevend/hypothese-genererend beschouwd.

Multivariate analyse

De hoeveelheid patienten in deze studie is te klein om een multivariate analyse op uit te voeren.

Interim analyse

Een interim analyse wordt niet uitgevoerd, omdat de studie 6 maanden duurt. Wel zal de data geregeld worden bijgewerkt.

ETHISCHE OVERWEGINGEN

Verklaring van Helsinki

De studie zal worden uitgevoerd volgens de principes van de “verklaring van Helsinki” (januari 2004) en in overeenstemming met de Wet op Medisch-wetenschappelijk onderzoek met Mensen (WMO). Tevens zal worden volgens de vigerende richtsnoeren (GMPz/GCP) worden gewerkt.

Recrutering en informed consent
De recrutering zal plaatsvinden door de behandelend arts (onderzoeker) tijdens een routine-onderzoek (follow-up na longtransplantatie). De patiënten krijgen standaard **twee weken de tijd** om na te denken over participatie, op verzoek kan dit worden verlengd. Gedurende deze tijd staat het ze vrij contact te zoeken met de behandelend arts, onderzoeker, onafhankelijk arts (Prof.Dr. H.A.M. Kerstjens) en alle anderen. Daarna zal de behandelend arts (onderzoeker) een nieuw gesprek met de vrijwilligers aangaan om uit te vinden of het onderzoek duidelijk is uitgelegd en zo nodig nogmaals duidelijk te maken. Tijdens dit gesprek kan het Informed Consent worden getekend. Het Informed Consent wordt vervolgens in een separaat studie-dossier bewaard.
Omdat de behandelend arts het recruteringsgesprek voert is er sprake van een afhankelijkheidssituatie. In het recruteringsgesprek en in de patiënteninformatie zal daaom duidelijk worden gemaakt dat ieder antwoord geenszins de verstandhouding tussen arts en patient zal verstoren en dat het geen effect heeft op de behandeling, dit om de belangen van de patient te waarborgen.

Groepsafhankelijke voordelen en risico-analyse
Dit onderzoek is het eerste in een reeks van onderzoeken. Het risico voor de patiënten is ingeschat op vrij laag gezien de ervaring die is opgedaan met ciclosporine in het algemeen en verneveling van ciclosporine bij longtransplantatie in nauwere zin. Daar waar de hiaten liggen in de optimalisering van de therapie voor longtransplantatie patiënten is in de voorkoming van systemische toxiciteit (als neuro- en nefrotoxiciteit) met behoud van effectieve immuunsuppressie in de getransplanteerde long. Volgens de nieuwste inzichten met verneveling van ciclosporine is het mogelijk om patiënten effectief te behandelen en de chronische afstoting te verminderen ten opzichte van de huidige – orale – therapie. Er is echter geen onderzoek gedaan naar de effectiviteit van inhalatie van ciclosporine als belangrijkste pilaar voor onderdrukking van het immuunsysteem met gelijktijdige voorkoming van nefrotoxiciteit.
Met dit onderzoek zal worden getracht om aan te tonen dat droog poeder inhalatie van ciclosporine ook effectief is in het verminderen van chronische afstoting (primair doel). Daarnaast wordt getracht om naast het verminderen van de chronische afstoting de nierfunctie in stand te houden (secundair doel). Ook wordt informatie gezocht over

inhaleerbaarheid van het poeder (depositie) en absorptie in de systemische circulatie vanuit de long (bloedspiegels). De voorstelling van de gang van zaken in de onderzoeken hierna is dat de nierfunctie een belangrijke rol zal gaan spelen: in hoeverre kan de dosis van de systemische therapie omlaag onder opbouwen van droog poeder inhalatie met gelijkblijvende mate van chronische afstoting en nierfunctie? Daarna zou een onderzoek kunnen volgen waarin de effectiviteit van droog poeder inhalatie als monotherapie na longtransplantatie. Dit zou ertoe moeten leiden dat ten opzichte van de huidige therapie de kwaliteit van het leven sterk toe zou moeten nemen. Er zou onder longtransplantatiepatienten minder sterfte komen door afname van de mate van chronische afstoting en de nierfunctie zou behouden kunnen blijven. Het is de bedoeling dat de gehele longtransplantie populatie van deze ontwikkeling zou moeten kunnen profiteren daar getracht wordt deze medicatie aan een geïnteresseerde partner te verkopen.

Compensatie

Verzekering

Ingevolge art. 7 van de Wet medisch wetenschappelijk onderzoek met mensen (Stbl. 1998, 161) is voor de deelnemende proefpersonen een verzekering afgesloten die de door het onderzoek veroorzaakte schade door dood of letsel van de deelnemende proefpersonen dekt. Deze verzekering voldoet aan de bepalingen van het Besluit verplichte verzekering bij medisch-wetenschappelijk onderzoek met mensen (Stbl. 2003, 266). De aan het onderzoek deelnemende proefpersonen zullen schriftelijk worden ingelicht over deze verzekering.

Tegemoetkomingen

Alle deelnemers in de studie zullen worden gecompenseerd voor de gemaakte reiskosten op basis van openbaar vervoer 2^{e} klasse.

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APPENDIX

Samenvatting

De snelle ontwikkelingen in de moleculaire biologie en biotechnologie van de afgelopen decennia hebben geleid tot de succesvolle introductie van een aantal biofarmaceutica, in het bijzonder therapeutische peptiden en eiwitten, op de geneesmiddelenmarkt. Biofarmaceutica zijn een speciale groep geneesmiddelen, omdat ze in het algemeen zowel chemisch als fysisch minder stabiel zijn dan de meeste kleine organisch gesynthetiseerde geneesmiddelmoleculen. Daarnaast zijn de meeste biofarmaceutica afhankelijk van een ruimtelijke structuur voor hun effectiviteit en veiligheid. Het behouden van die ruimtelijke structuur is daarom essentieel voor het ontwikkelen van effectieve en veilige producten. Zoals uitgelegd in de **algemene introductie** zijn een aantal biofarmaceutica (waaronder therapeutische eiwitten) uitstekend geschikt voor pulmonale toediening (inhalatie).
Het doel van de toediening per inhalatie kan het veroorzaken van een lokaal, lokaal en systemisch of alleen systemisch effect zijn. Er zijn verschillende typen inhalatoren beschikbaar om een aerosol te creëren. De aerosol kan worden gecreëerd vanuit een oplossing of een poeder. Wordt een aerosol gecreëerd vanuit een oplossing dan spreken we van verneveling. Verneveling is patiëntonvriendelijk, het kost veel tijd en vereist de beschikbaarheid van elektriciteit of perslucht. Daarnaast zijn de efficiëntie waarmee de aerosol wordt gecreëerd en de longdepositie slecht, waardoor relatief veel onnodig verlies optreedt. Een alternatief is de inhalatie van het geneesmiddel in poedervorm. Deze technologie kost minder dan een minuut voor volledige toediening. Daarnaast zijn de efficiëntie waarmee het aerosol wordt gecreëerd en de longdepositie in het algemeen hoger dan voor verneveling.
Omdat eiwitten vaak worden geproduceerd in waterige oplossingen vereist de toepassing in een droog poeder inhalator een droogstap. Die biofarmaceutica die afhankelijk zijn van hun tertiaire – lees: ruimtelijke – structuur voor hun activiteit, zoals eiwitten, moeten tijdens het droogproces worden gestabiliseerd. Hulpstoffen die aan de ene kant het eiwit fysiek fixeren en aan de andere kant de waterstofbruggen van het oplosmiddel kunnen vervangen zijn geschikt om biofarmaceutica te stabiliseren tijdens drogen en mogelijk ook tijdens opslag. Suikers zoals maltose, trehalose, sucrose en inuline, zijn voorbeelden van zulke hulpstoffen.

Verschillende droogmethoden zijn beschikbaar en enkele daarvan zijn in dit proefschrift beschreven: vriesdrogen, sproeidrogen en sproeivriesdrogen. Andere methoden, zoals bijvoorbeeld superkritische vloeistof extractie zouden ook in staat kunnen zijn om een poeder voor inhalatie in de juiste aerodynamische deeltjesgrootte te produceren.
Sproeivriesdrogen heeft als voordeel dat het eindproduct uit vaste deeltjes bestaat. Door gebruik te maken van de juiste procescondities kunnen deeltjes geproduceerd worden met de gewenste aerodynamische diameter tussen 1 en 5 µm. Sproeidrogen geeft ook de mogelijkheid een product met de gewenste aerodynamische grootte te produceren.
In dit proefschrift onderzochten wij in hoeverre biofarmaceutica verwerkt konden worden tot een droog poeder formulering. Daarnaast hebben we met een aantal producten een *proof-of-concept* studie uitgevoerd. Een aantal verschillende therapeutische peptiden en eiwitten hebben als modelstoffen gediend, die allen verschilden in mate van wateroplosbaarheid en molecuulgrootte. Daarnaast is een studie uitgevoerd met een stof die na inhalatie de lichaamseigen productie van een eiwit induceerde zodat de complexe formulering van een enzym kon worden vermeden.

In **Hoofdstuk 2** zijn drie verschillende productiemethoden vergeleken om een droog poeder formulering voor inhalatie te maken van cetrorelix. Cetrorelix is een hydrofiel peptide dat bestaat uit 10 aminozuren. Poeder met de gewenste deeltjesgrootte werd gemaakt via malen, sproeidrogen en sproeivriesdrogen. Alle methoden resulteerden in inhaleerbare deeltjes, maar de morfologie van de deeltjes verschilde per methode. Na verwerking van de poeders in adhesieve mengsels met lactose bleek dat het gesproeivriesdroogd poeder te fragiel was om de kinetische energie van de veel zwaardere lactosedeeltjes te weerstaan. De mengsels met gemalen en gesproeidroogd cetrorelix werden in twee verschillende inhalatoren getest: een capsule-inhalator en de Novolizer. Deagglomeratie in de capsule-inhalator vind plaats door de bewegende capsule, terwijl in de Novolizer een cycloon voor deagglomeratie zorgt middels botsingskrachten. *In vitro* testen toonden aan dat voor zowel de formulering met gemalen cetrorelix en de formulering met gesproeidroogd cetrorelix de Novolizer leidde tot efficiëntere deagglomeratie. Ook bleek dat naast het type inhalator, de morfologie van de deeltjes en daarmee de productiemethode van belang was:

gesproeidroogd cetrorelix leidde in beide inhalatoren tot een hoger percentage deeltjes die geschikt zijn voor inhalatie. Dit onderzoek leerde ons dat het adequaat ontwikkelen van inhalatiesystemen vraagt om een duale aanpak, waarin de specifieke combinatie van de inhaler met de poederformulering geoptimaliseerd worden.

De **hoofdstukken 3 en 4** beschrijven een studie met een hydrofoob peptide, ciclosporine A (CsA). CsA is een slecht wateroplosbaar cyclisch peptide van 11 aminozuren. Per inhalatie toegediend CsA zou toegepast kunnen worden bij chronische afstoting van getransplanteerde longen, een aandoening waarvoor tot op heden geen goede therapeutische behandelmogelijkheden zijn. In **Hoofdstuk 3** verkenden wij het gebruik van de *solid dispersion* technologie om een inhalatie poeder van CsA te ontwikkelen. *Solid dispersions* werden bereid door CsA op te lossen in tertiair butyl alcohol (TBA) en te mengen met een oplossing van water en een wateroplosbare hulpstof, inuline. Door middel van sproeivriesdrogen konden we een homogeen poeder van CsA en inuline produceren. Van dit poeder werd de oplossnelheid, de dispersie van CsA door de inuline matrix en het aerosolisatie gedrag onderzocht.
De *solid dispersions* losten sneller op dan het fysieke mengsel van CsA met inuline. Met Fourier Transformatie Infrarood Spectroscopie (FTIR) werd onderzocht hoe CsA ingebed zat in de hydrofiele matrix door gebruik te maken van het feit dat de conformatie van CsA in de hydrofiele matrix anders is dan in een hydrofobe matrix. Aangetoond werd dat de gemiddelde conformatie van alle CsA moleculen afhankelijk was van het gehalte, waarbij lage gehaltes een hoofdzakelijk hydrofiele conformatie kenden en hogere gehaltes een meer en meer hydrofobe conformatie bezaten. Blijkbaar worden bij hogere gehaltes meer en meer CsA moleculen geclusterd, mogelijk tot kleine deeltjes. Dit zou kunnen verklaren waarom de oplossnelheid afhankelijk was van het CsA gehalte in de *solid dispersion*.
Sproeivriesdrogen leidde tot deeltjes met een erg lage dichtheid en een erg hoog specifiek oppervlak van meer dan 100 m^2/gr. De deeltjes hadden een grote geometrische diameter, maar gecombineerd met de hoge porositeit resulteerde dit in een mediane aerodynamische diameter op gewichtsbasis (MMAD) van 3.39 µm. Vanwege de goede oplossnelheid en de uitstekende aerosoliseringseigenschappen concludeerden wij dat de

CsA solid dispersion met een gehalte van 50% de juiste parameters bezat voor gebruik bij longtransplantatiepatiënten.

Hoofdstuk 4 beschrijft de *in vivo* eigenschappen in de rat van het in hoofdstuk 3 beschreven CsA poeder voor inhalatie (iCsA). **Hoofdstuk 4A** richt zich op de farmacokinetiek en effectiviteit in het voorkomen van afstoting in een experimenteel longtransplantatiemodel in ratten. De farmacokinetiek van 10 mg/kg iCsA in ratten, gegeven middels insufflatie werd vergeleken met 10 mg/kg Neoral, oraal toegediend. Neoral is het huidige, commercieel beschikbare product. Hoewel iCsA en Neoral tot een vergelijkbare systemische beschikbaarheid leidden, resulteerde iCsA in een 109 keer hogere spiegel in de long vergeleken met Neoral.
Om de effectiviteit van iCsA te bestuderen werd in een transplantatiemodel de mate van afstoting vergeleken met Neoral. Het gebruikte longtransplantatiemodel resulteerde in volledige afstoting binnen 8 dagen na transplantatie als gevolg van de incompatabiliteit van de donor en ontvanger. iCsA bleek bij lagere dosis dan Neoral al effectief. Echter, in de bestudeerde dosering (5 mg/kg) werden microscopische reacties in de long waargenomen. Daarom werd in **Hoofdstuk 4B** onderzocht of lagere doses, de hulpstof inuline of insufflatie zelf ook tot vergelijkbare reacties zouden leidden. Bij een dosering van 0,8 mg/kg iCsA, week het aantal abnormaliteiten niet af ten opzichte van placebo.
Hoewel in hoofdstuk 4A werd aangetoond dat in het proefdier iCsA doses lager dan 5 mg/kg niet effectief waren in het remmen van de afstoting, is de veiligheidsstudie met een dosering van 0,8 mg/kg wel degelijk relevant, omdat deze dosering boven de dosering ligt waarvan in mensen al is aangetoond dat zij na inhalatie effectief kan zijn.
Voor een persoon van 60 kg zou een dosis van ongeveer 0,1 mg/kg al een effect geven. Zelfs in een zo lage dosering zou de CsA spiegel in de long vele malen hoger worden dan wanneer alleen Neoral oraal zou worden toegediend. Tegelijkertijd zou de plasmaspiegel van CsA, welke is gerelateerd aan het optreden van bijwerkingen zoals nierschade, niet noemenswaardig hoeven te stijgen. Daarom zijn wij van mening dat de behandeling van longtransplantatiepatiënten met chronische afstoting sterk verbeterd zou kunnen worden door iCsA.

Hoofdstuk 5 beschrijft de mogelijkheden om recombinant humaan deoxyribonuclease I (rhDNase), een eiwit van 37 kDa, te formuleren als een poeder voor inhalatie. In **Hoofdstuk 6** worden de farmaco-economische aspecten van een dergelijk product beschreven en wordt een vergelijking gemaakt met de thans commercieel beschikbare verneveling (Pulmozyme, 1 mg/ml rhDNase, 2,5 ml).
Verschillende processtappen om een poeder voor inhalatie van rhDNase te ontwikkelen werden bestudeerd. Naast de keuze van de stabiliserende hulpstof werden ook de effecten van procescondities op de inhaleerbaarheid onderzocht. Daarnaast hebben we met de meest geschikte formulering een *ex vivo* test uitgevoerd om de geschiktheid van het concept aan te tonen.
Van de onderzochte hulpstoffen sucrose, trehalose en inuline bleek sucrose niet geschikt als hulpstof. Chemisch gezien bleek trehalose de beste stabilisator, omdat het aantal dimere aggregaten niet toenam tijdens de stabiliteitstudie. Vervolgens hebben we inuline en trehalose formuleringen gesproeidroogd en gesproeivriesdroogd bij verschillende procescondities. Sproeidrogen bleek het beste proces te zijn omdat deeltjes met een MMAD van 2,3 µm geproduceerd konden worden. Ook bleek dat de formulering met inuline als hulpstof bij kamertemperatuur bewaard kon worden, in tegenstelling tot trehalose. Dit was waarschijnlijk gerelateerd aan de hoge glastransitietemperatuur van 157˚C van inuline. De inhalatie eigenschappen van de inuline formulering waren ook goed met een fijne deeltjesfractie (<5 µm) van ongeveer 40%. Daarom hebben we de effectiviteit van de inuline formulering getest met behulp van sputum van patiënten met cystische fibrose. Het bleek dat het poeder voor inhalatie significant zowel de viscositeit als de elasticiteit verlaagde. De conclusie was dat het goed mogelijk is om ook van grotere eiwitten als rhDNase een poeder voor inhalatie te ontwikkelen.

De in **Hoofdstuk 6** beschreven voordelen van droog poeder inhalatie ten opzichte van verneveling zijn *i)* een verbeterde therapietrouw, *ii)* een verbeterde longdepositie, *iii)* elektriciteit of perslucht zijn niet nodig, *iv)* een veel kortere toedieningstijd en *v)* de formulering hoeft niet gekoeld te worden. Deze voordelen zouden mogelijkerwijs kunnen leiden tot minder exacerbaties en ziekenhuisopnames. Daarmee zouden wellicht de kosten voor behandeling van cystische fibrose af kunnen nemen. De farmaco-economische analyse liet zien dat de kosten per gewonnen

levensjaar €19.110 zijn voor de verneveling, terwijl deze door de genoemde voordelen geschat worden op €12.100 voor de poederinhalatie. Dit betekent dat met droog poeder inhalatie ongeveer 40% lagere kosten gemaakt worden om eenzelfde effectiviteit te bereiken. Voor dure therapieën die een centrale rol spelen bij een therapie, zoals rhDNase bij cystische fibrose, kan het daarom nuttig zijn om in een vroegtijdig stadium te onderzoeken wat de farmaco-economische implicaties zijn van een bepaalde formuleringsstrategie.

In **Hoofdstuk 7** werd een alternatieve wijze onderzocht om een biofarmaceutische interventie tot stand te brengen: in plaats van een eiwit (enzym) te formuleren tot poeder voor inhalatie hebben we een natuurlijk substraat, een organisch molecuul, geformuleerd tot poeder voor inhalatie. Door het substraat toe te dienen in de longen wordt de lichaamseigen productie van het enzym geïnduceerd. Als modelsysteem hebben we heme oxygenase I (HO-1) als eiwit en als substraat hemine gebruikt, mede omdat in patiënten met chronisch obstructieve pulmonaire aandoening (COPD) was aangetoond dat dit enzymsysteem verlaagd is ten opzichte van gezonde vrijwilligers.
Vanwege de slechte oplosbaarheid van hemine bij pH 7 werd hemine opgelost in een alkalische oplossing die juist voor het sproeidrogen geneutraliseerd werd. Naast het formuleringsonderzoek werd ook een toedieningsmethode voor aerosolen aan kleine proefdieren onderzocht, waarbij de dieren door de natuurlijke ademhaling het aerosol inhaleren. Met het ontwikkelde hemine poeder hebben werd aangetoond dat het enzymsysteem op een dosisafhankelijke wijze kon worden geïnduceerd.
In dit hoofdstuk is aangetoond dat het ook mogelijk is om relatief simpele organische moleculen aan te bieden om een effect te bewerkstelligen wat gebaseerd is op een biofarmaceutische interventie. Het voordeel hiervan is dat er geen ingewikkelde formuleringen nodig zijn voor het betreffende eiwit. Daarnaast is een nieuwe toedieningsmethode van aerosolen geïntroduceerd. Deze methode is uniek omdat enerzijds iedere droogpoeder inhalator aangesloten zou kunnen worden, en anderzijds omdat de methode het mogelijk maakt vergelijkend onderzoek te doen met een aantal formuleringen in een vroegtijdig stadium van de ontwikkeling. Daarnaast maakt de methode gebruik van de natuurlijke ademhaling van de proefdieren om het poeder te inhaleren.

Tot slot, alle in dit proefschrift onderzochte geneesmiddelen konden worden geformuleerd als een poeder voor inhalatie. Afhankelijk van de eigenschappen van het specifieke molecuul konden we specifieke formuleringstechnologieën aanwenden om de ruimtelijke structuur te bewaren tijdens het droogproces, opslag en reconstitutie. In bepaalde gevallen zou men kunnen volstaan met aanbieden van een organisch molecuul zonder dat het biofarmaceutisch molecuul zelf geformuleerd hoeft te worden. Omdat de effectiviteit van de toediening per inhalatie afhankelijk is van de aerodynamische eigenschappen van de aerosol (en daarmee van het poeder), is er een noodzaak om deze eigenschappen in proefdieren te testen via de pulmonaire route. In dit proefschrift zijn insufflatie en blootstelling via de neus getest. Insufflatie is vooral geschikt voor studies waarin een kwantitatieve relatie met de dosering onderzocht wordt, zoals farmacokinetische studies, terwijl blootstelling via de neus gebruikt kan worden voor het vaststellen van een *proof-of-concept* en het vergelijken van aerodynamische eigenschappen van verschillende formuleringen.

APPENDIX

Publications, patents and presentations

related to this thesis

PUBLICATIONS

2009 Zijlstra G.S., Wolting M.J., Petersen A.H., Prop J., Uges D.R.A., Kerstjens H.A.M., Van Der Bij, W., Frijlink H.W. *The efficacy of a new pulmonary cyclosporine A powder formulation in the prevention of transplant rejection in rats.* J Heart Lung Transplant, 28(5):486-92

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PATENTS

2005 Zijlstra G.S., Van Drooge D.J., Hinrichs W.L.J., Dickhoff B.H.J., Frijlink H.W. *Process for preparing formulations of lyophilic active substances by spray freeze drying*. US Patent No. 11/269,954.

PRESENTATIONS

2007 *Dry powder inhalation of cyclosporine a in rats: pharmacokinetics and effectiveness in rejection prevention in lung transplantation* – 19th BOOT congress NTV, Zeewolde, the Netherlands

2005 *Cyclosporine A – Dry Powder Inhalation,* Medical aerosol think tank, Utrecht, the Netherlands.

2005 *Inhalable inulin-based solid dispersion containing Cyclosporine A*. Belgian-Dutch Biopharmacy Society, Ghent, Belgium.

2003 *Formulation of cetrorelix acetate as a powder for inhalation – comparison of micronization techniques*, Belgian-Dutch Biopharmacy Society, Groningen, the Netherlands.

2002 *Pulmonary drug delivery: the lung as a delivery route for peptides and proteins*, Medical aerosol think tank, Utrecht, the Netherlands.

2002 *Deoxyribonuclease I in inhalation therapy – the formulation of a stable and inhalable powder*, Belgian-Dutch Biopharmacy Society, Leuven, Belgium.

2002 *Effective powder deagglomeration principle enables the application of classical dry powder formulation techniques for peptides in inhalation*, Respiratory Drug Delivery 8, Tucson, United States (poster).

APPENDIX

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APPENDIX

Curriculum vitae

Gerrit Zijlstra is geboren (19 mei 1974) en getogen in Bolsward. Na de HAVO en het VWO op het RKSG Titus Brandsma College afgerond te hebben, is hij in 1994 Farmacie gaan studeren aan de Rijksuniversiteit in Groningen, vanaf 1995 gecombineerd met een specialisatie in Farmaceutische Technologie. Het afstudeeronderzoek, afgerond in 2000, is deels uitgevoerd aan het *Institut für Spektrochemie und angewannte Spektroskopie* in Dortmund, Duitsland onder begeleiding van dr. Bert Schoonen en dr. Michael Heise. Het afstudeeronderzoek betrof het ontwikkelen van een non-invasieve glucose sensor op basis van infrarood spectroscopie. Na zijn afstuderen in 2000 is Gerrit een promotieonderzoek gestart bij de vakgroep Farmaceutische Technologie & Biofarmacie aan de Rijksuniversiteit Groningen onder begeleiding van Prof. dr. Erik Frijlink op droog poeder inhalatie van eiwitten en peptiden. Tijdens het promotie-onderzoek heeft Gerrit de apothekersopleiding in 2002 succesvol afgerond. In 2007 is Gerrit gaan werken bij Disphar International BV in Baarn, eerst als Research & Development Associate en later als Projectmanager voor generieke productontwikkeling. In 2009 heeft Gerrit de overstap gemaakt naar Novartis Pharma BV als Medical Scientific Liaison op respiratoir gebied binnen de afdeling Scientific Operations. Gerrit is getrouwd en heeft twee kinderen.